METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF AMBRISENTAN IN TABLET DOSAGE FORM BY HPLC

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MASTER OF PHARMACY

In

(Pharmaceutical Analysis)

Submitted By

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This is to certify that the project entitled, **"METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF AMBRISENTAN IN TABLET DOSAGE FORM BY HPLC "** submitted **by Regd. No.261430012** in partial fulfillment for the award of degree of **Master of Pharmacy (Pharmaceutical Analysis).** The project was carried out at IDEAL ANALYTICAL LAB, PONDYCHERRY and C.L.Baid Metha college of Pharmacy, Chennai-97 under my supervision during the academic year 2015-2016.

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DECLARATION

I hereby declare that this dissertation entitled, **"METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF AMBRISENTAN IN TABLET DOSAGE FORM BY HPLC"** has been originally carried out by me under the guidance and supervision of **Dr. N.Ramalakshmi, M.Pharm., Ph.D**. Department of Pharmaceutical Analysis, C.L.Baid Metha College of Pharmacy, Chennai – 97 during the academic year 2013-2015. The work embodied in this thesis is original, and is not submitted in part or full for any other degree of this or any other University.

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"Success is how high you bounce when you hit bottom"

"If you can dream it, you can do it"

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ABBREVIATIONS

AUC	Area under concentration
Conc.	Concentration
FDA	Food and drug administration
gms	Gram
hrs	Hours
ICH	International conference on harmonization
LOD	Limit of detection
LOQ	Limit of quanitation
mg	Milli gram
mL	Milli liters
Mins	Minutes
mm	Milli meter
ng	Nano gram
NLT	Not less than
nm	Nano meter
NMT	Not more than
ODS	Octadecylsilane
PAH	Pulmonary arterial hypertension
Psi	Pascal per square inch

RP-HPLC	Reverse phase high performance liquid chromatography
RSD	Relative standard deviation
Rt	Retention time
S. No.	Serial number
Std.Dev	Standard deviation
USP	United states pharmacopoeia
UV	Ultra violet
µL	Micro liters
µm	Micro meters
µg (or) mcg	Micro gram
^{0}C	Degree Celsius
%	Percentage

1. INTRODUCTION

Pharmaceutical analysis plays a vital role in the Quality Assurance and Quality control of bulk drugs. Analytical chemistry involves separation, identification, and determining the relative amounts of components in a sample matrix; Pharmaceutical analysis is a specialized branch of analytical chemistry that derives its principles from various branches of sciences like physics, microbiology, nuclear science, and electronics etc. Qualitative analysis is required before a quantitative analysis can be undertaken.

Analytical method development and validation is a good research in the field of Pharmaceutical analysis, utilized to determine the drug content in bulk and pharmaceutical dosage forms and in biological fluids like blood, serum, urine etc. In view of the industrial scenario and literature, it was noted that chromatographic techniques like HPLC, LC–MS/MS methods have created revolutionary precision and accuracy in quantification of drugs in Formulation and in Biological fluids even at low concentration.

Need for pharmaceutical Analysis

- New drug development.
- Method Validation as for ICH Guidelines
- Research in Pharmaceutical Sciences
- Clinical Pharmacokinetic Studies

1.1 Chromatographic Techniques

Recently, the IUPAC has defined chromatography as; "Methods used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while other moves. The stationary phase may be a solid or a liquid supported on a solid or a gel, and may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid".[1]

M.T Swett coined the term "chromatography" to describe the process of separating colorful leaf pigments in a column made of chalk.[2] Chromatography is a non-destructive technique used for separating a mixture of compounds into individual components using a stationery phase and mobile phase.[3] Chromatography is used routinely in almost every laboratory for a large number of tasks. These range from the separationof mixtures on an analytical as well as preparative scale, purification and pre concentration of an analyte to controlling the progress of a chemical reaction.

Classification of Chromatographic Techniques

- According to the nature of stationary and mobile phase
  - Gas Solid Chromatography
  - Gas Liquid Chromatography
  - Solid Liquid Chromatography
  - Liquid Liquid Chromatography

- According to mechanisms of separation, chromatographic methods are divided into
  - Adsorption chromatography
  - Partition chromatography
  - Ion exchange chromatography
  - Ion pair chromatography
  - Size exclusion or gel permeation chromatography
  - Affinity chromatography
  - Chiral phase chromatography

1. Adsorption chromatography

The principle of separation is adsorption. Separation of components takes place because of the difference in affinity of compounds towards stationary phase. This principle is seen in normal phase as well as reverse phase mode, where adsorption takes place.

2. Partition chromatography

When two immiscible liquids are present, a mixture of solutes will be distributed according to their Partition co efficient .When a mixture of compounds are dissolved in the mobile phase and passed through a column of liquid stationery phase, the component which is more soluble in the stationery phase travels slower. The component which is more soluble in the mobile phase travels faster. Thus the components are separated because of the differences in their partition co- efficient. No two components have the same partition co-efficient for a particular combination of stationery phase, mobile phase and other conditions. The stationery phase as such cannot be a liquid

3. Ion exchange chromatography

The principle of separation is ion exchange, which is reversible exchange of functional groups. In ion exchange chromatography, an ion exchange resin is used to separate a mixture of similar charged ions. For cations, a cations exchange resin is used. For anions, an anion exchange resin is used. In ion exchange chromatography, the stationary solid phase of the chromatographic column (or sheet or other device) commonly consists of a resin with covalently attached anions or cat ions.[4]When the sample, dissolved in water or another liquid, is run over the column, sample ions of the opposite charge are attracted to the stationary ions of the column by electrostatic forces and weakly bind to them. Adsorbed

sample components are then eluted by application of a salt gradient which gradually desorbs the sample molecules in order of increasing electrostatic interaction with the ions of the column.

4. Ion pair chromatography

In ion pair chromatography, a reverse phase column is converted temporarily into ion exchange column by using ion pairing agents like pentane or hexane or heptanes or octane sulphonic acid sodium salt, tetraethyl or tetra methylhydroxide etc.

5. Size exclusion or gel permeation chromatography

In this type of chromatography, a mixture of components with different molecular sizes is separated by using gel. The gel used acts as molecular sieve and hence mixture of substances with different molecular sizes is separated. Soft gels like dextran are also used.

6. Affinity chromatography:

Affinity chromatography uses the affinity of the sample with specific stationary phases. This technique is mostly used the field of biotechnology, micro biology, biochemistry etc.

7. Chiral phase chromatography

Separation of optical isomer can be done by using chiral stationary phases different principles operate for different types of stationary phases and different samples. The stationary phases are used for this type of chromatography are mostly chemically bonded silica gel.

In chromatographic separation, HPLC and HPTLC methods have widely been exploited in pharmaceutical analysis because of its simplicity, precision, accuracy and reproducibility of results.[1]

1.2 INTRODUCTION TO HPLC

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.

HPLC allows the use of very small particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. Its simplicity, high specify and wide range of sensitivity makes it ideal for analysis of many drugs in both dosage forms and in biological fluid.

1.3 TYPES OF HPLC TECHNIQUES

A. Based on modes of separation: There are two modes, normal phase mode and reverse phase mode[5.]

i) Normal phase mode: In this mode**,** the stationary bed is strongly polar in nature (e.g., silica gel), and the mobile phase is nonpolar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

ii) Reveres phase mode Reverse-phase chromatographyis the inverse of Normal phase mode. The stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar

the material is, the longer it will be retained.Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of vanderwaals dispersion forces. Since most of the drugs and pharmaceuticals are polar in nature, they are not retained for a longer time and eluted faster which is advantageous. Different columns used are ODS (octadecylsilane), C_{18}, C_8, C_4 etc.

B. Based on elution technique

Isocratic separation In this technique the same mobile phase combination is used throughout the process of separation, the same polarity or elution strength is maintained through the process.

Gradient separation In this technique, a mobile phase combination of low polarity or elution strengths is used followed by gradually increasing the polarity or elution strengths.

C. Based on the scale of operation:-

Analytical HPLCIn this technique only analysis of the samples are done. Recovery of the samples for reusing is normally not done, since the sample used very low. Ex. µgm quantities.

Preparative HPLCIn this technique individual fraction of pure compounds can be collected using fraction collector. The collected samples are reused. Ex. Separation of few grams of mixtures by HPLC.

D. Based on the type of analysis

Qualitative analysis It is done to identify the compound, detect the presence of impurity, to find out the number of components etc... This is done by using retention time values.

Quantitative analysisItis doneto determine the quantity of the individual or several components in a mixture. This is done by comparing the peak area of the standard and sample.

1.4 PRINCIPLE OF HPLC

The principle of separation in normal phase mode and reverse phase mode is adsorption and/or partition.

ADSORPTION

When a mixture of components is introduced in to a HPLC column they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the stationary phase travels slower. The component which has less affinity towards the stationary phase travels faster. Since no two components have the same affinity towards the stationary phase the components are separated

PARTITION

When a mixture of compounds are dissolved in the mobile phase and passed through a column of liquid stationery phase, the component which is more soluble in the stationery phase travels slower. The component which is more soluble in the mobile phase travels faster. Thus the components are separated because of the differences in their partition co-efficient. No two components have the same partition co-efficient for a particular combination of stationery phase, mobile phase and other conditions.

1.4 INSTRUMENTATION OF HPLC

The main components of HPLC system are as follows

a) Mobile phase reservoir

b) pumps

c) Sample mixing units

d) Sample injectors

e) Column

f) Detectors.

g) Recorders and injectors

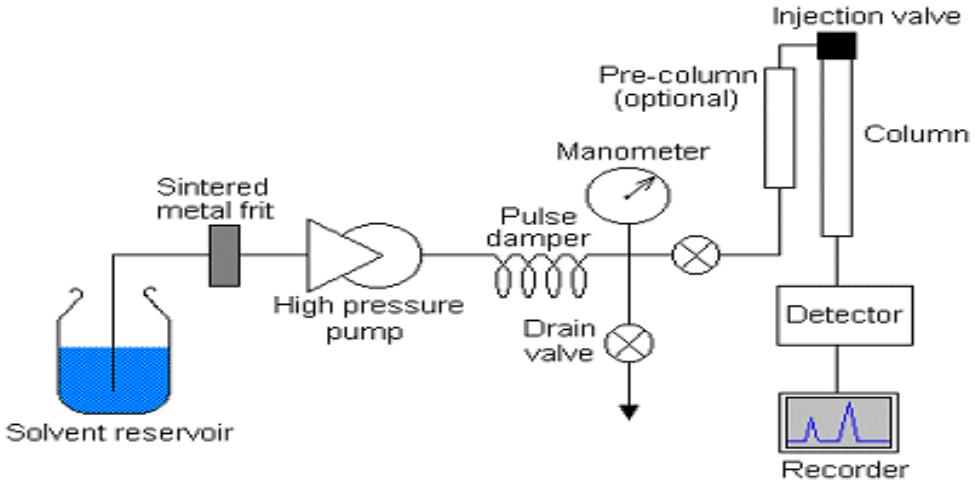


Fig No 1: Schematic diagram of HPLC

a) Mobile phase Reservoirs The modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs each of which contains 200-1000ml of a solvent. The mobile phase are often equipped with a mean of removing dissolved gasses usually oxygen or nitrogen. These bubbles cause band spreading, in addition they often interfere with performance of the detectors. Often the systems also contain a means of filtering dust and particulate matter from the solvent to prevent these particles from damaging the pumping systems or clogging systems. This treatment removes gases as well as suspended matter. A separation that employs a single solvent of constant composition is termed as isocratic

elution. Frequently separation efficiency is greatly enhanced by gradient elution. Here two difference solvent systems that differ significantly on polarity are employed.

b) Pumps The most important component of HPLC in solvent delivery systems is the pumps because its performance directly affects the retention time reproducibility and detector sensitivity.

Three types of pumps each with its own set of advantages are encountered namely.

- Reciprocating pumps
- Displacement –type pumps
- Pneumatic pumps

i) Reciprocating Pumps

They usually consist of chamber in which the solvent is pumped by the back and forth motion of a motor drive piston. The two ball check valves which open and close alternately control the flow of solvent in to and (close alternately control the flow of solvent in to and) out of a cylinder track with the piston.

ii) Displacement Pumps

Displacement pump consist of large, syringe like chambers equipped with a plunger that is activated by a screw driven mechanism powered by a stepping metor. The output is pulse free.

iii) Pneumatic Pumps

The pneumatic pumps, the melphis contained in a compatible container housed in a vessel that can be pressurized by compressed gas. Pumps of this type are in expensive

and pulse free[6-7], they suffer from limited capacity and pressure output as well as a dependence of flow rate on solvent viscosity and column back-pressure. In addition they are not amiable to gradient elution and are limited to pressure less than about 2000psi.

c) Flow Control and Programming Systems

As part of their pumping systems, many commercial instruments are equipped with computer controlled devices for measuring the flow rate by determining the pressure drop across a restrictor located at the pump outlet.

d) Sample Mixing Units

Mixing unit is used to mix solvents in different proportions and pass through the column. They are Low pressure mixing chamber which uses helium for degassing solvents and High pressure mixing chamber does not require helium for degassing solvents.

e) Sample Injector

Often the limited factor in the precision of liquid chromatographic measurement lies in the reproducibility with which samples can be introduced in to columns packing. Exaggerated by band broadening which accompanies overloading columns. These volumes used must be minuscule a (Thus, the volumes used must be mini) of a ml to perhaps 500ml.It is convenient to be able to introduce the sample without depressurizing the system.

f) Columns

HPLC columns are ordinarily constructed from smooth-bore stainless steel tubing, although heavy-walled glass tubing is occasionally encountered.

Analytical columns

The majority of HPLC columns range in length from 10-30cm. Normally columns are straight with added length when needed, being gained by coupling two or more columns together occasionally called columns are encountered

The inside diameter of liquid column is often 2-5mm.The most common particles size of packed columns is 5-10µm .the most common columns currently in use is one that is 15-25cm in length,4.6mm inside diameter ,and packed with 5µm particles. Columns of this type contain 4000-6000plates/meter.

Guard columns

The guard columns have very small quantity of adsorbent and improve the life of the analytical columns. It also acts as a prefilter to remove particulate matter and any other material. Guard column has the same material as that of analytical column but does not contribute to any separation.

g) Column thermostats

For many applications close control of column temperature is not necessary and columns are appareled at combined temperature often. However better chromatograms are obtained by monitoring column temperature constant to few degree centigrade. Most modern commercial instruments are now equipped with column heaters that control, column temperature to a few tenths of degree from near obtained to 100-150 ^{o}c. Columns may also be fitted with water jackets fed from a constant temperature bath to give precise temperature control.

h) Column material

The columns are made up of either stainless steel glass polyethylene, PEEK (poly ether ether ketone). Most widely used are stainless steel which can with stand high pressure. Latest ones are PEEK columns whose length varies from 5-30cm, diameter ranges from 2-5mm and particle size from 1µ-20µ.Particle should be spherical and uniform in nature. Porous materials are used. Surface area of 1gm of stationary phase provides surface area ranging from 100-860 mts.

i) Functional group

The functional group present in stationary phase depends on the type of chromatographic separation in normal phase mode it contain following groups

- C_{18} –octadecylSilane (ODS) column
- C_8-octylcolumn
- C_4-butyl column
- CN-cyano column
- NH_2-amino column

j) DETECTORS

The function of detector in HPLC is to monitor the mobile phase as it emerges through the column

Types of detectors

i) **Absorbance detector**A ‘Z’ shaped flow through cell for absorbance measurement on elements from a chromatographic column in order to minimize extra column band

broadening the volume of such a cell is kept as small as possible. The typically volumes are limited to 1-10µl and cell length to 2-10mm here intensity of the solvent system are stored in a computer memory and ultimately recalled for the calculation of absorbance.

ii) **UV detector**UV detector is based on the light absorption characteristics of sample. Two types of this detector are, one is the fixed wavelength detector which operates at 254nm, and other is variable wavelength detector which can be operated from 190nm to 400nm.

iii) Refractive index detector This is a nonspecific or universal detector. This is not much used for analytical applications because of low sensitivity and specificity.

iv) Flourimetric detector Flourimetric detector is based on fluorescent radiation emitted by some class of compound. In most cases fluorescence is absorbed by a photoelectric detector located at 90ºto the excitation beam. The simplest detectors employ a mercury excitation sources and one or more filters to isolate a band of emitted radiation. This detector has more sensitivity and specificity.

v) Conductivity detector Based upon electrical conductivity, the response is recorded. This detector is used when the sample has conducting ions like anions and cat ions

vi) Amperometric detector Amperometric detector is based on the reduction or oxidation of the compounds when a potential is applied. The diffusion current recorded is proportional to the concentration of the compound eluted this is applicable when compounds have functional group which can be either oxidized or reduced this is a highly high sensitive detector.

vii) PDA detectorPDA detector is a recent one which is similar to UV detector which operates from 190-600nm. Radiations of all wavelengths fall on the detector simultaneously. The resulting spectrum is 3-D or three dimensional plot of response Vs time Vs wavelength.

k) Recorders and integrators Recorders are used to record the responses obtained from detectors after amplification, if necessary. They record the base line and all the peaks obtained, with respect time to time. Retention time for all the peaks can be found out from such recordings, but the area of individual peaks cannot be known.

Integrators are improved variation of recorders with some data processing capabilities. They can be recorded the individual peaks with retention time, height and width of peaks, peak area, percentage of area, etc.

1.5 GENERAL INFORMATION ON HPLC METHOD DEVELOPMENT

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling.

The important factors, which are to be taken into account to obtain reliable quantitative analysis, are

a) Careful sampling and sample preparation.

b) Precise sample injection.

c) Appropriate choice of the column.

d) Choice of the operating conditions to obtain the adequate resolution

e) Reliable performance of the recording and data handling systems.

f) Suitable integration/peak height measurement technique.

g) The mode of calculation best suited for the purpose

h) Validation of the developed method.

Separation goals:

The goals of HPLC separation need to be specified clearly include the use of HPLC to isolate purified sample components for spectral identification or quantitative analysis. It may be necessary to separate all degradants or impurities from a product for reliable content In quantitative analysis, the required levels of accuracy and precision should be known (a precision of 1 to 2% is usually achievable). Whether a single HPLC procedure is sufficient for raw material or one or more for mutations and/or different procedures are desired for formulations. When the number of samples for analysis at one time is greater than 10, a run time of less than 20 minutes often will be important.

Getting Started On Method Development

One approach is to use an isocratic mobile phase of some average solvent strength (50%) organic solvent. A better alternative is to use a very strong mobile phase first (80-100%) then reduce %B as necessary.

The initial separation with 100% B results in rapid elution of the entire sample, but few groups will separate. Decreasing the solvent strength shows the rapid separation of all components with a much longer run time, with a broadening of latter bands and reduced retention sensitivity.

Goals that are to be achieved in method development are briefly summarized in below table in order of decreasing importance but may vary with analysis requirements.

GOAL	COMMENTS
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	<5-10 min is desirable for routine procedures.
Quantitation	2% for assays; 5% for less-demanding analyses 15% for trace analyses.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratios.
Solvent consumption	Minimum mobile-phase use per run is desirable.

Table No:1 Suitable parameters for HPLC method development

1.6 VALIDATION

The word **"validation"** means "Assessment" of validity or action of validity or action of providing effectiveness'.

Definitions

FDA defines validation as "establish the documented evidence which provides a high of assurance that a specific process will consistently produce a product of predetermined Specifications and quantity attributes".

WHO action of providing that, any procedure, process, equipment, material, activity, or system actually leads to the expected results.

EUMGP define validation as "action of proving in accordance with the principle of Good manufacturing practice (GMP), that any material, activity or system actually lead to expected result".

AUSTRALIAN GMP defines validation as "the action of proving that any material, process, activity, procedure, system, equipment or mechanism and intended results".

1.7 METHOD VALIDATION GUIDELINES

1.7.1 FDA guidelines

The FDA published the draft on "Guidance for Industry on Analytical Procedures and Method Validation" to aid pharmaceutical companies in meeting the code of federal regulations requirement [FDA, 2000]. This guidance, when approved, supersedes the FDA Guidance for Industry on Submitting Samples and Analytical Data for Method Validation [FDA, 1987]. According to the FDA, analytical methods are categorized into four tests: identification, testing for impurities (quantitative and limit), assay (dissolution, content, potency), and specific tests. Recently, the Centre for Drug Evaluation and Research (CDER), a division of the FDA, also issued guidance for validation of bio analytical methods [FDA-CDER, 2001].

1.7.2 ICH guidelines

The ICH was initiated around 1990. It is an important regulatory initiative to standardize regulatory requirements between the European Community, Japan and the United States. Recognizing the benefits of having consistent international requirements, ICH developed a number of guidelines that have been recognized in these countries. The two main documents of ICH guidelines pertaining to analytical method validation are:

(i) Q2A: Text on Validation of Analytical Procedures [ICH-Q2A, 1995]

(ii) Q2B: Validation of Analytical Procedure Methodology [ICH-Q2B, 1997] (6)

1.7.3 USP guidelines

The USP [USP, 2006] categorizes analytical methods into four types of tests: quantisation of major components of drug product, testing for impurities (quantitative and limit), performance characteristics and identification tests. USP refers to the same definitions of ICH Q2A and Q2B recommendations for procedures on meeting validation requirements.

Table 2: Comparison of Validation Parameters Required for HPLC Assay Methods

ICH Guidelines	USP Guidelines	FDA Guidelines
Accuracy	Accuracy	Accuracy
Precision	Precision	Precision
Repeatability	--	Repeatability
Inter-day precision	--	Inter-day precision
Reproducibility	--	Reproducibility
Specificity	Specificity	Specificity
Limit of detection	Limit of detection	Limit of detection
Limit of quantification	Limit of quantification	Limit of quantification
Linearity	Linearity	Linearity
Range	Range	Range
--	Ruggedness	--
Robustness	Robustness	Robustness
System suitability	System suitability	System suitability(7)

1.8 ANALYTICAL METHOD VALIDATION

Method validation is the process for establishing that performance characteristics of the analytical method are suitable for the intended application. Chromatographic methods need to be validation before first routine use. To obtain the most accurate results, all of the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, detection and data evaluation, using the same matrix as that of the intended sample. The validity of an analytical method can only be verified by laboratory studies. All validation experiments used to make claims or conclusions about validity of the method should be documented in report.

Types of analytical procedures to be validated

- Identification test for impurities
- Quantitative test for impurities
- Limit test control of impurities
- Quantitative test for the active moiety in samples of drug substance or drug product, or other selected components (s) in the drug product.
- Dissolution testing.

1.9 VALIDATION PARAMETERS AS PER ICH & FDA GUIDELINES

1.9.1 Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. For formulation assay, a sample matrix may include impurities, degradation products, excess raw materials, or excipients. The chromatographic method should be

specific and sensitive as required for all known relevant degradation products and/or impurities.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures

This definition has the following implications:

Identification : to ensure the identity of an analyte.

Purity Tests : to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

1.9.2 Linearity:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. A range of standards should be prepared containing at least 5 different concentrations of analyte, which are approximately evenly spaced, and span 80-120% of the label claim.

Acceptance criteria:

Correlation coefficient should be not less than 0.9990.

% of y-intercept should be ± 2.0. % of RSD for level 1 and level 5 should be not more than± 2.0

1.9.3 Range:

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The specified range is in general derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

The following minimum specified ranges should be considered:

- For the assay of a drug substance or a finished (drug) product: normally from 80 to120 percent of the test concentration.
- For content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified.
- For dissolution testing: +/-20 % over the specified range.

1.9.4 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found.

The accuracy may be determined by application of analytical method to an analyte of known purity (example: reference standard) and also by comparing the results of the method with those obtained using an alternate procedure that has been already validated.

The true value for accuracy assessment can be obtained in several ways and the value found as:

One method is to compare the results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known.

Secondly, accuracy can be assessed by analyzing a sample with known concentrations and comparing the measured value with the true value as supplied with the material.

Third method if certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible. At each recommended level studied, replicate samples are evaluated. Accuracy should be assessed using a minimum of determinations over a minimum of 3 concentrations (3 replicates each of the total analytical procedure). Validation and as well the accuracy should be within the range 98-102%.

1.9.5 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample

under the prescribed conditions. Precision of an analytical procedure is usually expressed the variance, standard deviation of coefficient of variation of a series of measurement.

Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

For a good method precision, the intra-day and inter-day precisions should be within the acceptance criteria of % RSD ≤2.0 respectively.

1.9.5.1 Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

1.9.5.2 Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

1.9.5.3 Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

1.9.6 Limit of detection & Limit of quantification

The LOD of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantified as an exact value. The LOQ of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. The typical methods recommended by ICH to determine the LOD and LOQ are:

(i) Signal-to-noise ratio: The LOD and LOQ can be expressed as a concentration at a specified signal-to-noise ratio obtained from samples spiked with analyte.

(ii) Standard deviation of the response and the slope of the calibration curve(s) at levels approximating the LOD: LOD was defined as 3.3σ/S and LOQ as 10σ/S, where S is the slope of the calibration curve and σ is the standard deviation that can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of the regression lines.

1.9.7 Ruggedness

Ruggedness is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of test conditions such as different laboratories, analysis, instruments, reagent lots, elapsed assay times, temperature, days etc.

It can be expressed as lack influence of the operation and environmental variable on the test results of the analytical method.

1.9.8 Robustness

It is measure of capacity of an assay to remain unaffected by small but deliberate variation in method parameters and provide an indication of its reliability in normal usage degradation and variation in chromatography columns; mobile phase and inadequate method development are common causes of lack of robustness.

1.10 Parameters in Chromatography

A good resolution of components is achieved by optimization of various parameters such as Resolution (Rs), number of theoretical plates (N), capacity factor (k'), and peak asymmetry factor (AF), etc.[12]

- **Retention time (R_t)**

Retention time is the difference in time between the point of injection and appearance of peak maxima. Retention time is the time required for 50% of a component to be eluted from a column. Retention time is measured in minutes or seconds. Retention time is also proportion to the distance moved on a chart paper; which can be measured in cm or mm.

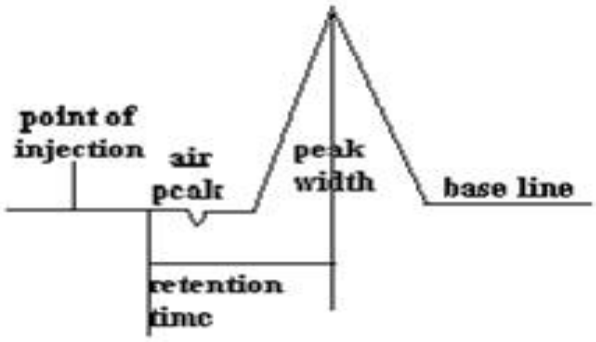


Figure No 4: Retention time

- **Retention volume (R_v) -** Retention volume is the volume of carrier gas required to elute 50% of the component from the column. It is product of retention time and flow rate.

Retention volume = retention time X flow rate

- **Separation factor (S):-**

Separation factor is the ratio of partition co-efficient of the two components to be separated. It can be expressed and determined by using the following equation:

S= Kb/KA=Ka/Kb= (tb-to)/ (ta-to)

Where to =retention time of unretained substance

Kb, Ka=partition coefficients of b and a

Tb, ta=retention time of substance band a

S= depends on liquid phase, column temperature

If there is more difference in partition coefficient between two compounds, the peaks are far apart and the separation factors are less.

- **Capacity factor (k')**

It is the measure of how well the sample molecule is retained by the column during an isocratic separation. It is affected by solvent composition, separation and aging and temperature of separation.

$K' = V_1-V_0 / V_0$

Where V_1 = retention volume at apex of the peak

V_0 = void volume of system where an unretained component elutes

- **Column efficiency (N)**

It is called as number of theoretical plates. It measures the band spreading of a peak. When band spread is smaller, the number of theoretical plate is higher. It indicates to good column and system performance.

$N = 16 (R_t/W)^2$

Where, R_t = Retention time

W = Width of peak

Width of peak can be calculated using different methods of calculations. Column efficiency should be measured under ideal conditions of column, mobile phase and flow rate used. All these factors affect column efficiency.

- **Selectivity (α)**

It measures relative retention of two components. Selectivity is the function of chromatographic surface (column), melting point and temperature.

$\alpha = K'_2/K'_1 = V_2 - V_0/V_1 - V_0$

- **Resolution**

It is a measure of separation between adjacent peaks in chromatogram. The amount of separation between 2 peaks is a function of 2 parameters. As the difference between retention time of peak increases, the separation also increases. Resolution is proportional to difference in retention times of peaks. The amount of separation depends on that portion of width of each peak which is nearest to the adjacent peak. As half width of peak increases, the amount of separation between peaks decreases and resolution also decreases.

$Rs = 2(Rt_2 - Rt_1) / W_2 - W$

Where, Rt_1, Rt_2 – retention times of components

W_1, W_2 – width of two adjacent peaks

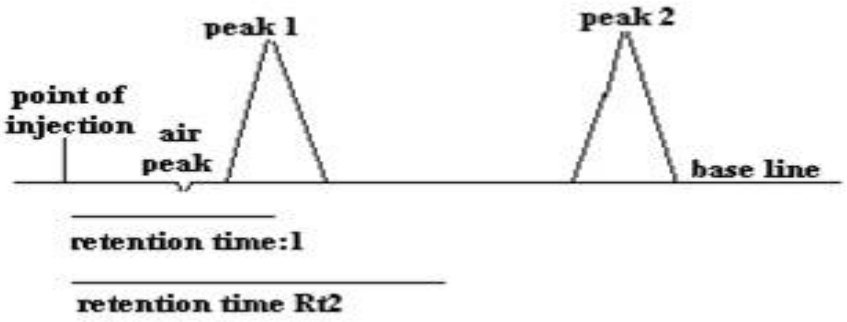


Figure No 5: Resolution oftwo peaks

- **Peak asymmetric or Tailing Factor**

A_F = b/a (at 10% or 5% of peak height)

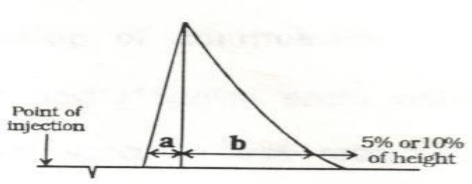


Figure No 6 :Tailing Factor

- **Ideal Valves**

K' = 2-10 results in good separation

α = 2 good peak separation results

N = 3000-10,000 plates/ column

R_S = 1.5 A good baseline separation occurs

AF = 0.95 – 1.05

If K increases – R_S increases but peak becomes broadens

K decreases – R_S decreases but peak becomes sharper

If N decreases – R_S decreases because peak width broadens

N increases – R_S increases because peak width narrows

If α increases – R_S increases because peak moves in relation to anterior.[13]

Tailing factor establishes the maximum permissible asymmetry of the peak.

T = W / 2 × F

Where: T = Tailing factor

W= Peak width at 5% of peak height

F = time from width start point at of peak height to retention time (Rt)

- **Mean Value (Average)**

It is a measure of the location of the data. It is simply the arithmetic average of data add them all up and divide by the number of data points. The mean of a set of data is usually considered the best estimate of the value.[14]

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n}(x_i)$$

Where X =mean, n=no of value

- **Standard Deviation**

It is a measure of the spread of data around the sample mean – a precision measure. It is always in the same units as the mean. Like the mean, it is considered an estimate of the population standard deviation.

$$s(x_i) = \sqrt{\frac{1}{n-1}\cdot\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

Where S (X_i) =standard deviation, X=mean, n=no of values

- **Relative Standard Deviation**

Relative standard deviation is a measure of the spread of data in comparison to mean of the data. It is simply the standard deviation divided by the mean value.

$$RSD = \frac{s(x_i)}{\bar{x}} \quad (absolute\ or\ \%)$$

2. LITERATURE REVIEW

1. RamakrishnaNirogi*et.al.*,[15] developed a LC-ESI-MS/MS method for quantification of ambrisentan in plasma and application to rat pharmacokinetic study. The analyte and the internal standard (armodafinil) were extracted from plasma by acetonitrile precipitation and they were separated on a reversed-phase C(18) column with a gradient program. The assay exhibited a linear dynamic range of 1-2000 ng/mL for ambrisentan in plasma. Acceptable precision (<10%) and accuracy (100 ± 8%) were obtained for concentrations over the standard curve range.

2. Douaja M *et.al.*,[16] developed a method for rapid determination of ambrisentan enantiomers by enantio selective liquid chromatography using cellulose-based chiral stationary phase in reverse phase mode. Six chiral columns were tested in a reversed-phase system. Excellent enantiomeric separation with the resolution more than 2.5 was achieved on Chiralcel OZ-3R (cellulose 3-chloro-4-methylphenylcarbamate) using mixture of 20 mM sodium formate (pH 3.0) with acetonitrile (55:45., v/v).

3. **Klivicky*et.al.*,**[17] developed a LC/MS/MS method for determination of ambrisentan, midazolam and its metabolite in human plasma. After solid phase extraction on a Strata-X (Phenomenex) SPE column, the analytes were separated on a Synergi MAX-RP (150×4.6 mm, 4 µm) (Phenomenex) analytical column using isocratic elution with acetonitrile/5mM ammonium acetate 41:59 (v/v) as a mobile phase.Detection was achieved using tandem mass spectrometry on a triple quadrupole mass spectrometer. Ambrisentan-d3,midazolam-d5 and 13 C3-1-hydroxymidazolam were used as internal standards. The calibration curves were linear between 2.03 and 150 ng/ml for MDL (r^2 = 0.9993) and OH-MDL (r^2 = 0.9987) and between 5.40 and 400 ng/ml for ABT (r^2 = 0.9974).

4. Jun Xia *et.al.,*[18] developed a method for Synthesis and in vitro evaluation of ambrisentan analogues as potential endothelin receptor antagonists. A series of novel 2-[(4, 6-dimethylpyrimidin-2-yl) oxy]-3, 3-diphenyl butyric acid derivatives were synthesized and evaluated for their antagonistic activity for endothelin-1-induced contraction in rabbit aorta.

5. Johanna Weiss *et.al.,*[19] made an in vitro study on Differential modulation of the expression of important drug metabolising enzymes and transporters by endothelin-1 receptor antagonists ambrisentan and bosentan. LS180adenocarcinoma cells were treated for four days with bosentan or ambrisentan (1–50 μM), the positive control rifampicin, or medium only (negative control). For evaluation of bosentan also HuH-7 human hepatoma cells were used. Comparable to rifampicin, bosentan was a moderate to strong inductor for all cytochrome P450 isozymes and ATP-binding cassette transporters tested, and it also induced organic anion transporting polypeptides. In contrast, ambrisentan only weakly induced some of the genes investigated in LS180 cells. These findings corroborate the in vivo finding that bosentan is much more prone to drug interactions than ambrisentan.

6. JudyW.M.Cheng*et.al.,*[20] demonstrated the role of Ambrisentan for the Management of Pulmonary Arterial Hypertension. The available clinical data suggest that ambrisentan is an effective and well-tolerated therapy for the management of PAH

7. Ronald J.Oudiz*et.al.,*[21] made a study on Long-Term Ambrisentan Therapy for the Treatment of Pulmonary Arterial Hypertension. After 2 years of ambrisentan exposure, the mean change from baseline in 6-min walk distance was improved for the 5-mg (23 m., 95% confidence interval: 9 to 38 m) and 10-mg (28 m., 95% confidence interval: 11 to 45 m) groups. Two years of ambrisentan treatment was associated with sustained improvements in exercise capacity and a low risk of clinical worsening and death in patients with PAH. Ambrisentan was generally well tolerated and had a low risk of aminotransferase abnormalities over the 2-year study period.

8. **J. Dupuis *et.al*.,**[22] investigated the role of Endothelin receptor antagonists in Pulmonary arterial hypertension. The endothelin (ET) system, especially ET-1 and the ETA and ETB receptors, has been implicated in the pathogenesis of pulmonary arterial hypertension (PAH). Together with prostanoids and phosphodiesterase 5 inhibitors, ET receptor antagonists have become mainstays in the current treatment of PAH. Three substances are currently available for the treatment of PAH. One of these substances, bosentan, blocks both ETA and ETB receptors, whereas the two other compounds, sitaxsentan and ambrisentan, are more selective blockers of the ETA receptor.

9. MichaelD.McGoon*et.al*.,[23]made a study on ambrisentan therapy in patients with pulmonary arterial hypertension who Discontinued Bosentan or Sitaxsentan Due to Liver Function Test Abnormalities. Patients who previously discontinued bosentan and/or sitaxsentan due to LFT abnormalities received ambrisentan, 2.5 mg qd, for 4 weeks followed by 5 mg/d for 8 w.eeks. The primary end point was the incidence of aminotransferase levels more than three times ULN considered by the investigator to be related to ambrisentan and resulting in drug discontinuation. Secondary end points included aminotransferase levels more than five times ULN requiring drug discontinuation and more than three times ULN requiring dose reduction, as well as changes in 6-min walk distance (6MWD

10. Nazzareno Galiè*et.al*.,[24] conducted Randomized, Double-Blind, Placebo-Controlled, Multicenter, Efficacy (ARIES) tests in patients receiving ambrisentan for PAH therapy and concluded that Ambrisentan improves exercise capacity in patients with pulmonary arterial hypertension and it is is well tolerated and is associated with a low risk of aminotransferase abnormalities.

11. BrianCasserly*et.al.*,[25] conducted a study on role of Ambrisentan in the treatment of pulmonary arterial hypertension and concluded that Ambrisentan appears to be a safe and efficacious treatment for PAH patients in WHO functional class II or III.

12. Rebecca Spence *et.al.*,[26] reported the pharmacokinetics and Safety of Ambrisentan in combination with Sildenafil in Healthy Volunteers. The pharmacokinetic interaction between sildenafil, and ambrisentan, was studied in a 2-period crossover study in 19 healthy volunteers, with ambrisentan exposure (AUC0-∞) and Cmax determined over 24 hours for a 10-mg dose of ambrisentan alone and again after 7 days of sildenafil 20 mg 3 times daily. The AUC0-∞ and Cmax for sildenafil and N-desmethyl sildenafil (active metabolite) were determined over 24 hours for a 20-mg dose of sildenafil alone and again after 7 days of dosing with ambrisentan 10 mg once daily. There was no clinically relevant pharmacokinetic interaction between ambrisentan and sildenafil or N-desmethyl sildenafil.

13. **Gennyne Walker *et.al.*,**[27] conducted the pharmacokinetics and pharmacodynamics of warfarin in combination with ambrisentan in healthy volunteers. Twenty two healthy subjects were administered a single dose of racemic warfarin 25 mg alone and after 8 days of ambrisentan 10 mg once daily. Assessments included exposure (AUC0–last) and maximum plasma concentration (Cmax) for R and S-warfarin, and International Normalized maximum observerved value (INRmax) and area under the curve ($INR_{AUC(0–last)}$).Ambrisentan had no significant effects on the (AUC0–last)of R-warfarin or S-warfarin. In addition, coadministration of warfarin did not alter ambrisentan steady-state pharmacokinetics. Adverse events were infrequent, and there were no bleeding adverse events.

14. HartmutVatter*et.al.*,[28] demonstrated the role of ambrisentan as a Non-peptide Endothelin Receptor Antagonist, concluded that because of convenient physiological and pharmacological properties and its good tolerability ambrisentan will probably play an

essential role in the therapy of diseases, for which a potent ETA-receptor antagonist with a moderate selectivity is the most customized approach.

15. **Ori Ben-Yehuda *et.al.*,**[29] conducted a study on Long-Term Hepatic Safety of Ambrisentan in Patients With Pulmonary arterial hypertension. No evidence of an association between ambrisentan therapy and hepatotoxicity was identified

16. **Rebecca Spence *et.al.*,**[30] reported the Pharmacokinetics and Safety interactions of Ambrisentan in Combination With Taldafil in Healthy Volunteers. Single-dose PK of ambrisentan (10 mg) and its metabolite, 4-hydroxymethyl ambrisentan, were determined in the absence and presence of multiple doses of tadalafil (40 mg QD). Similarly, single-dose PK of tadalafil (40 mg) were evaluated in the absence and presence of multiple doses of ambrisentan (10 mg QD). In the presence of tadalafil, ambrisentan maximum plasma concentration (C_{max}) was similar and systemic exposure ($AUC_{0-\infty}$) was slightly decreased compared with ambrisentan alone.

17. M. Halank*et.al.*,[31] conducted a study on patients with ambrisentan therapy and concluded that Ambrisentan Improves Exercise Capacity and Symptoms in Patients with Portopulmonary Hypertension. No significant changes in blood gas analysis and liver function tests (aspartate aminotransferase, alanine aminotransferase, total bilirubin, and international normalized ratio) during therapy with ambrisentan were detectable.

18.**J.Craig Hartman *et.al.*,**[33] Evaluated the endothelin receptor antagonists ambrisentan, darusentan, bosentan, and sitaxsentan as substrates and inhibitors of hepatobiliary transporters in sandwich-cultured human hepatocytes, and concluded that Bosentan and Sitaxsentan ,but not Darusentan and Ambrisentan inhibit human hepatic transporters which provides a potential mechanism for the increased hepatotoxicity.

3.1 AIM & OBJECTIVE OF WORK

The drug analysis plays an important role in the development of new analytical methods, their manufacture and the therapeutic use. Pharmaceutical industries rely upon quantitative chemical analysis to ensure that the raw materials used and the final product obtained meets the required specification. The components monitored include chiral or achiral drug process impurities, residual solvents excipents such as preservatives, degradation products, extractable and leachable matterfrom containers and closuresetc.

Ambrisentan is indicated for the treatment of patients with pulmonaryarterial hypertension (PAH) classified as World Health organisation (WHO) functionalclass (FC) II and III, to improve exercise capacity. Ambrisentan, is a propanoic acid-classan endothelin receptor antagonist that isselective for the endothelin type-A (ETA) receptor. It is a Bio pharmaceutics Classification System Type II drug.It has a low potential for drug-drug interactions when compared to other endothelin receptor antagonists and requires only once-daily administration.

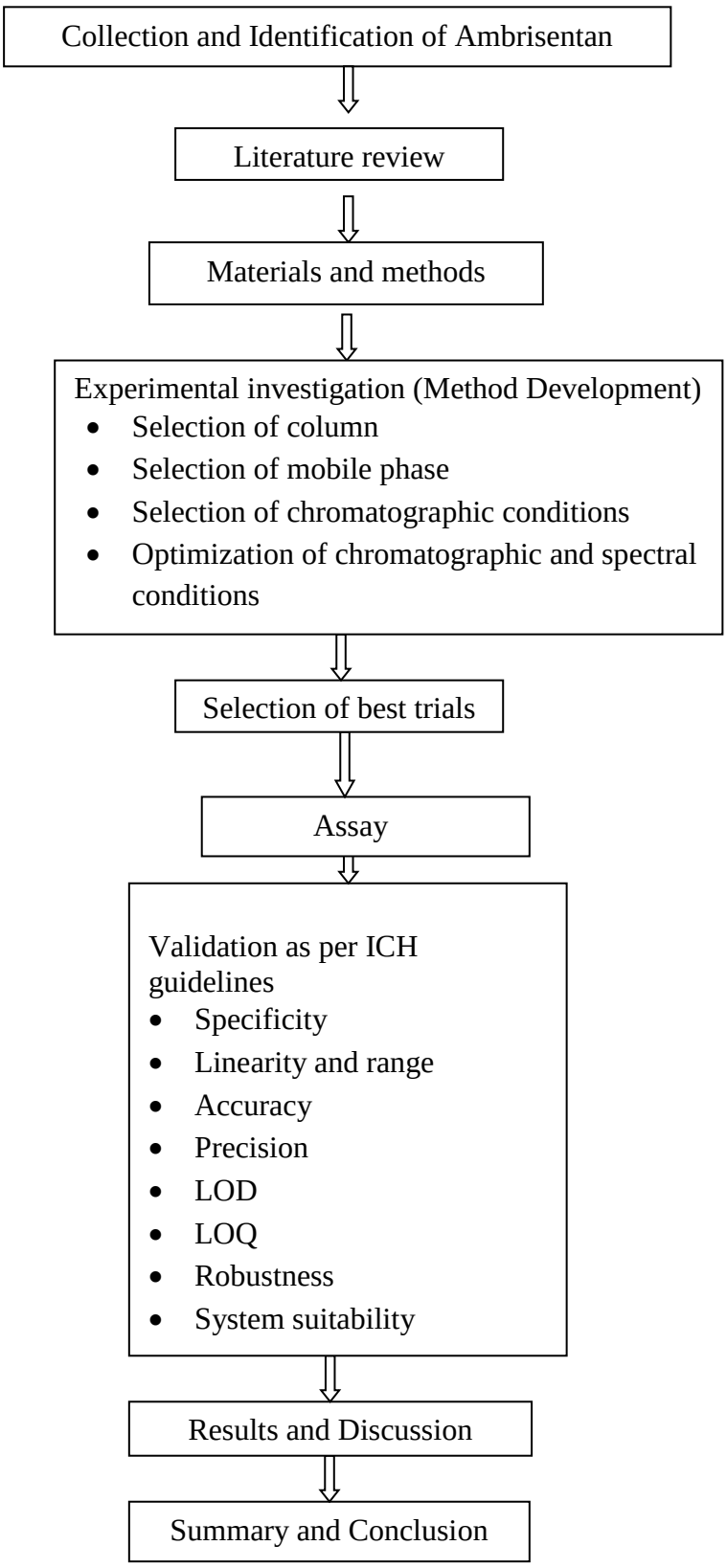
The extensive literature survey reveals that no method has been developed for the estimation of Ambrisentan in tablet dosage form. The objective of the present work is to develop a specific, precise, accurate, linear, simple, rapid and validated RP-HPLC method forAmbrisentan.

The specific aim of the research was

- To develop a RP-HPLC method for determination of Ambrisentan in tablet dosage form.

- Validate the optimized method in accordance with ICH guidelines for the intended analytical application.

3.2 PLAN OF WORK

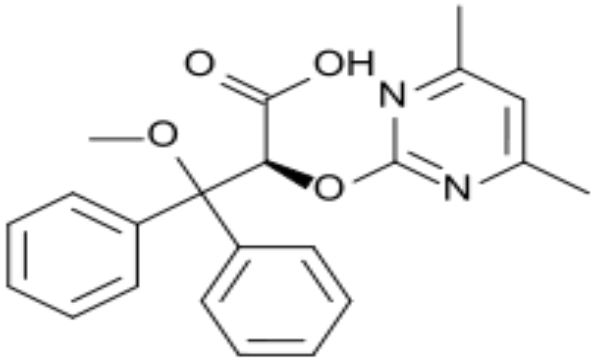


4. DRUG PROFILE[35]

Ambrisentan

Chemical formula :$C_{22}H_{22}N_2O$.

Structure :



Chemical name :(2S)-2-[(4, 6-dimethylpyrimidin-2-yl) oxy]-3-methoxy- 3, 3-diphenylpropanoic acid

Molecular weight : 378.421gm/mol

Description : white to off-white crystalline substance.

Solubility : Insoluble in water (0.06 mg/ml) and soluble in aqueoussolutions of high pH

PKa : 4.0

Category :Endothelin type A (ETA)-selective receptor antagonist

Dose :5 or 10 mg in tablet

Mechanism of action[36]

Ambrisentan is an orally active, propanoic acid-class, endothelin receptor antagonist (ERA) that is selective for the endothelin type A (ETA) receptor. Selective inhibition of the ETA receptor inhibits phospholipase C-mediated vasoconstriction and protein kinase C-

mediated cell proliferation, while preserving nitric oxide and prostacyclin production, cyclic GMP- and cyclic AMP-mediated vasodilation, and endothelin-1 (ET-1) clearance that is associated with the endothelin type B (ETB) receptor.

Pharmacokinetics [36]

Absorption

The absolute bioavailability of ambrisentan is not known. Ambrisentan is absorbed rapidly in humans. After oral administration, maximum plasma concentrations (Cmax) of ambrisentan typically occur between 539-1147 ng/ml around 1.5 hours post dose under both fasted and fed conditions.[37]Cmax and area under the plasma concentration-time curve (AUC) increase dose proportionally over the therapeutic dose range. Steady-state is generally achieved following 4 days of repeat dosing.

Distribution

Ambrisentan is highly plasma protein bound. The in vitro plasma protein binding of ambrisentan was on average 98.8% and independent of concentration over the range of 0.2 – 20 microgram/mL. Ambrisentan is primarily bound to albumin (96.5%) and to a lesser extent to alpha1-acid glycoprotein.

The distribution of ambrisentan into red blood cells is low, with a mean blood:plasma ratio of 0.57 and 0.61 in males and females, respectively.

Metabolism

Ambrisentan is excreted largely unchanged (45.6% of the dose). Ambrisentan is glucuronidated via several UGT isoenzymes (UGT1A9S, UGT2B7S, and UGT1A3S) to form ambrisentanglucuronide (13%). Ambrisentan also undergoes oxidative metabolism mainly by CYP3A4 and to a lesser extent by CYP3A5 and CYP2C19 to form 4-hydroxymethyl ambrisentan (21%) which is further glucuronidated to 4-hydroxymethyl ambrisentanglucuronide (5%). The binding affinity of 4-hydroxymethyl ambrisentan for the

human endothelin receptor is 65-fold less than ambrisentan. Therefore at concentrations observed in the plasma (approximately 2% relative to parent ambrisentan), 4-hydroxymethyl ambrisentan is not expected to contribute to pharmacological activity of ambrisentan.

Excretion

Ambrisentan and its metabolites are eliminated primarily in the bile following hepatic and/or extra-hepatic metabolism with approximately 66% of the oral dose excreted in the faeces, the majority of which is unchanged ambrisentan (41% of the dose). Approximately 22% of the administered dose is recovered in the urine following oral administration with 3.3% being unchanged ambrisentan. Plasma elimination half-life in humans ranges from 13.6 to 16.5 hours.

Half-life: 15 hr terminal; 9 hr effective

INDICATIONS

Ambrisentan is indicated for the treatment of:

- Idiopathic pulmonary arterial hypertension (PAH),
- Pulmonary arterial hypertension associated with connective tissue disease (PAH-CTD),
- In patients with WHO functional class II, III or IV symptoms.

CONTRAINDICATIONS

Ambrisentan is contraindicated in

- **Pregnancy**

  Ambrisentan is a category X drug and must not be given to pregnant women. Pregnancy must be avoided during treatment and for at least 3 months following cessation of treatment with this drug.

- Women of child-bearing potential who are not using reliable contraception. Women must not become pregnant for at least 3 months after stopping treatment with ambrisentan.
- Patients with severe hepatic impairment (with or without cirrhosis) .
- Patients with baseline values of hepatic aminotransferases (aspartate aminotransferase [AST] and/or alanine aminotransferase [ALT]) greater than 3 times the Upper Limit of Normal (ULN)
- Patients who exhibit or may exhibit hypersensitivity to ambrisentan

Possible side effects[38]

Ambrisentan is expected to provide optimal results in the treatment of PAH. However, like all drugs, there are certain side effects that can be expected when undergoing Ambrisentan drug treatments, the most common of which is the occurrence of a headache. Ambrisentan patients also face the risk of developing liver function abnormalities; thus far, there has been a low incidence of this side effect in clinical trial testing.

❖ **Hepatic**

Hepatic side effects have included dose-dependent liver injury manifested primarily by elevation of serum aminotransferases (ALT or AST), but sometimes accompanied by abnormal liver function (elevated bilirubin). The combination of aminotransferases greater than 3 times the upper limit of normal and total bilirubin 2 times the upper limit of normal is a marker for potentially serious hepatic injury.

❖ **Hematologic**

Hematologic side effects have included decreases in hemoglobin concentration and hematocrit. Post marketing reports have included anemia. There have been post marketing

reports of decreases in hemoglobin concentration and hematocrit that have resulted in anemia requiring transfusion. In the long-term open-label extension of the two pivotal clinical studies, mean decreases from baseline (ranging from 0.9 to 1.2 g/dL) in hemoglobin concentrations persisted for up to 4 years of treatment.

❖ **Cardiovascular**

Cardiovascular side effects have included peripheral edema, palpitations, and flushing. Fluid retention has been reported during post marketing use of Ambrisentan. Additional post marketing reports have included heart failure (associated with fluid retention).

❖ **Respiratory**

Respiratory side effects have included dyspnea, nasal congestion, sinusitis, and nasopharyngitis.

❖ **Gastrointestinal**

Gastrointestinal side effects have included abdominal pain and constipation. Post marketing experience has included nausea and vomiting.

❖ **Nervous system**

Nervous system side effects have included headache.

5. MATERIALS & EQUIPMENTS

6.1 List of materials used for method development

For the determination of Ambrisentan in tablet dosage form by RP-HPLC method the following instruments were used.

Table:3 List of Instruments

S. No.	Name	Model	Manufacturer
1	HPLC with VWD detector	LC1200	Agilent Technologies
2	PH meter	-	Eutech
3	Sonicator	-	Remi Instruments
4	Analytical balance	BSA2245-CW	Sortorius

For the determination of Ambrisentan in tablet dosage form by RP-HPLC method the following chemicals were used.

Table:4 List of Chemicals

S. No.	Name	Grade	Manufacturer
1	Potassium Dihydrogen Orthophosphate	AG	Fischer scientific
2	Phosphoric acid	AG	Fischer scientific
3	Acetonitrile	HPLC	Fischer scientific
4	Milli Q water	HPLC	In House production

For the determination of Ambrisentan in tablet dosage form by RP-HPLC method the following Standard drugs were used as a reference standard.

Table: 5 List of Standard drugs

S. No.	Name	Specification purity %	Procured from
1	Ambrisentan WS	99.5	Gift Sample from MSN Laboratories Ltd, India

The following marketed formulation was used for the determination of purity of Ambrisentan in tablet dosage form by RP-HPLC method,

Table: 6 Marketed formulation

Brand name	Label claim	Manufacturing company
ENDOBLOC	Ambrisentan (5mg) + Excipients (qty specified)	MSN LABORATORIES LTD,INDIA

For the determination of Ambrisentan in tablet dosage form by RP-HPLC method the following apparatus were used.

Table:7 List of Apparatus

S.No.	Apparatus	Manufacturing
1	Beakers	Borosil
2	Pipettes	Borosil
3	Measuring cylinders	Borosil
4	Volumetric flasks	Borosil
5	Glass rods	-
6	Spatula	-
7	Rubber bulbs	-
8	Filter papers(0.45µ)	Whatmann

6. ANALYTICAL METHOD DEVELOPMENT

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling. The important factors, which are to be taken into account to obtain reliable quantitative analysis, are

a). Careful sampling and sample preparation.

b). Precise sample injection.

c). Appropriate choice of the column.

d). Choice of the operating conditions to obtain the adequate resolution of mixture

e). Reliable performance of the recording and data handling systems.

f). Suitable integration/peak height measurement techniques.

g). the mode of calculation best suited for the purpose.

h). Validation of the developed method.

Preparation of Standard drug solution

Approximately 25mg of Ambrisentan was weighed and transferred into50mL volumetric flask, diluted to 30 ml with mobile phase and sonicated for 15minutes. Then the volume was made up to 50mL with mobile phase and filtered through 0.45µ membrane filter. Further 5mL of above solution was diluted to 25 ml and mixed to get a concentration of 100 µg/ml.

6.1 Analytical Method development trials

Trial No.1

Mobile phase preparation

The mobile phase was prepared by mixing HPLC grade methanol and water in the ratio 50:50 v/v. Then it was sonicated and filtered through 0.45µ membrane filter.

Chromatographic conditions

Standard solution of Ambrisentan was loaded in the vial, injected and run for 30 min. The HPLC parameters were set in the method as follows.

- Column :Kromosil C_{18}, 250×4.6 mm, 5µm
- Column temperature : Ambient
- Mobile Phase : Methanol: water (80:20 v/v)
- Flow rate : 1mL/min
- Injection volume : 10µL
- Wave length : 210nm
- Run time : 30min

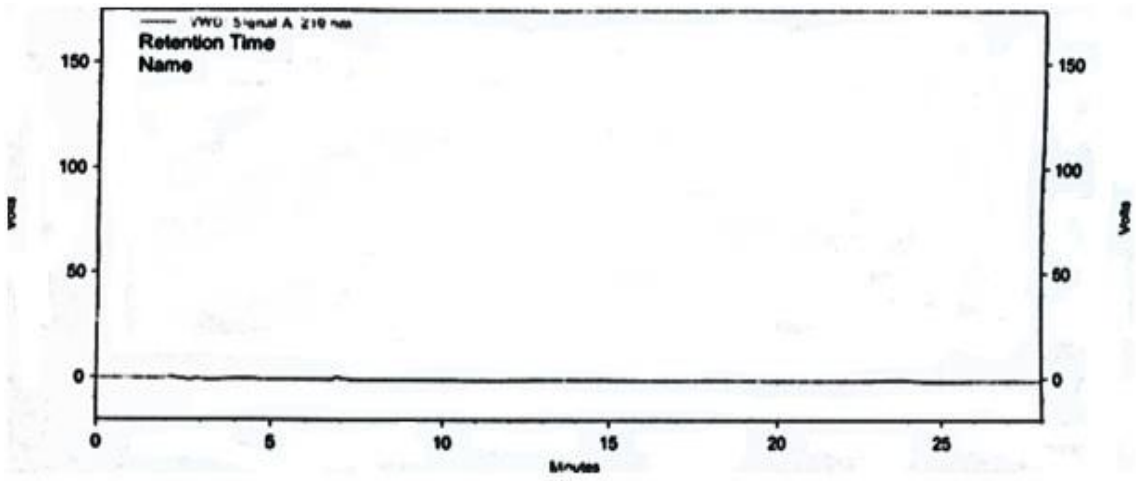


Figure 7: HPLC chromatogram for first trial

Table: 9 Results for trial 1

Drug	Retention time	Tailing factor	Theoretical plates
Ambrisentan.	------	------	------

Discussion

No peak was eluted so the mobile phase has been changed for next trail.

Trial No.2

Mobile phase preparation

The mobile phase was prepared by mixing HPLC water and Acetonitrile in the ratio 50:50 v/v. Then it was sonicated and filtered through 0.45µ membrane filter.

Chromatographic conditions

Standard solution of Ambrisentan was loaded in the vial, injected and run for 30min. The HPLC parameters were set in the method as follows.

- Column : KromosilC_{18},250×4.6mm, 5µm
- Column temperature : Ambient
- Mobile Phase : Acetonitrile: water(50:50 v/v)
- Flow rate : 1mL/min
- Injection volume : 10µL
- Wave length : 210nm

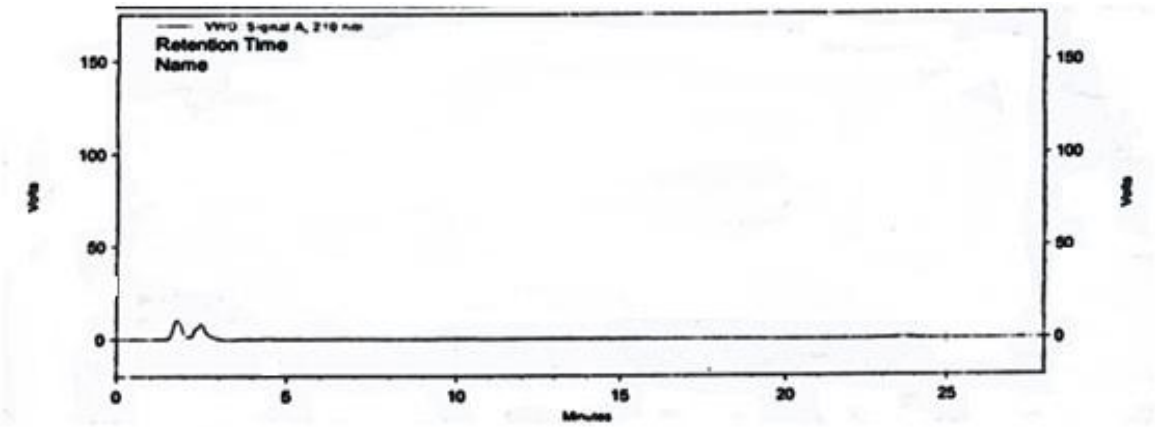


Figure8: HPLC chromatogram for second trial

Table: 10 Results for trial 2

Drug	Retention time	Tailing factor	Theoretical plates
Ambrisentan.	------	------	------

Discussion

No peak was eluted so the mobile phase has been changed for next trail.

Trial No.3

Preparation of Buffer

2.72 gms of potassium dihydrogen orthophosphate is dissolved in 1000 ml of water and pH is adjusted to 3.5±0.05 with dilute ortho-phosphoric acid, then sonicated and filtered through 0.45µ membrane filter.

Mobile phase preparation

The mobile phase was prepared by mixing mobile phase A:B in the ratio of 50:50 v/v.The mobile phase A is potassium dihydrogen orthophosphate buffer and mobile phase Bis Acetonitrile: water (90:10 v/v), then sonicated and filtered through 0.45µmembrane filter.

Chromatographic conditions

Standard solution of Ambrisentan was loaded in the vial, injected and run for 30min. The HPLC parameters were set in the method as follows.

- Column : KromosilC_{18}, 250×4.6 mm, 5µm
- Column temperature : 35^0C
- Mobile phase : A:B(50:50 v/v)
  - Mobile phase A : Potassium dihydrogen orthophosphate buffer (pH 3.5)
  - Mobile phase B : Acetonitrile: water (90:10 v/v)
- Flow rate : 1mL/min
- Injection volume :10µL
- Wave length : 210nm

.

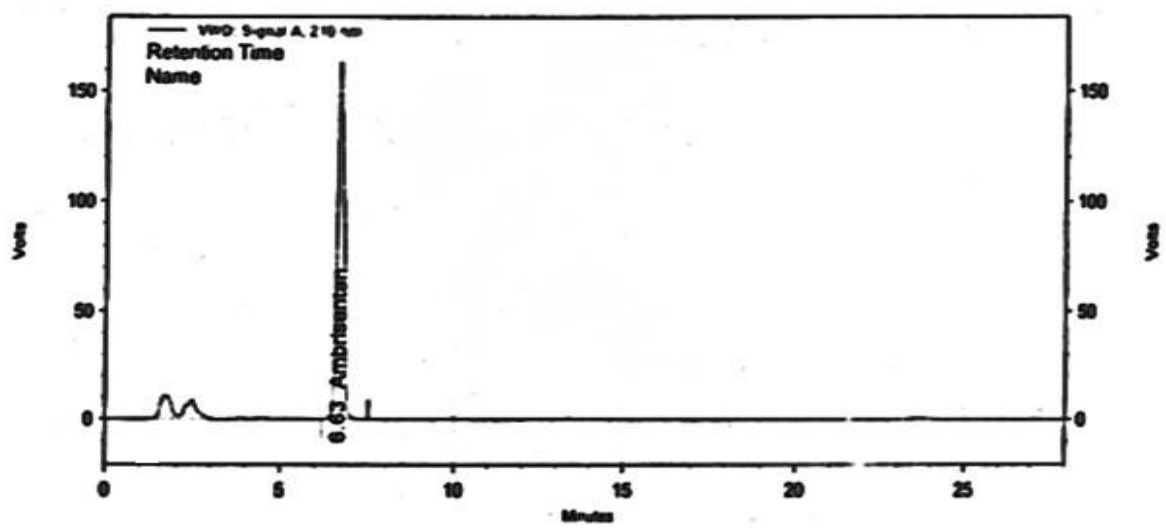


Figure 9: HPLC chromatogram for third trial

Table: 11 Results for trial 3

Drug	Retention time	Tailing factor	Theoretical plates
Ambrisentan.	6.63	2.97	10512

Discussion

The analyte peak was well eluted but noise was observed in the base line. The remaining suitability parameters were also not within the limits.

Trial No.4

Preparation of Buffer

2.72 gms of potassium dihydrogen orthophosphate is dissolved in 1000 ml of water and pH is adjusted to 3.5±0.05 with diluted phosphoric acid , then sonicated and filtered through 0.45µ membrane filter.

Mobile phase preparation

The mobile phase was prepared by mixing mobile phase A:B in the ratio of 40:60 v/v. The mobile phase A is potassium dihydrogen orthophosphate buffer and mobile phase B is Acetonitrile:Water (90:10v/v), then sonicated and filtered through 0.45µ membrane filter.

Chromatographic conditions

Standard solution of Ambrisentan was loaded in the vial, injected and run for 10min. The HPLC parameters were set in the method as follows.

- Column : Kromosil C_{18}, 250×4.6mm, 5µm
- Mobile phase : A:B(40:60 v/v)
  - Mobile phase A : Potassium dihydrogen orthophosphate buffer (pH 3.5)
  - Mobile phase B : Acetonitrile: water (90:10 v/v)
- Column temperature : 35^0C
- Flow rate : 1mL/min
- Injection volume : 10µL
- Wave length : 210nm

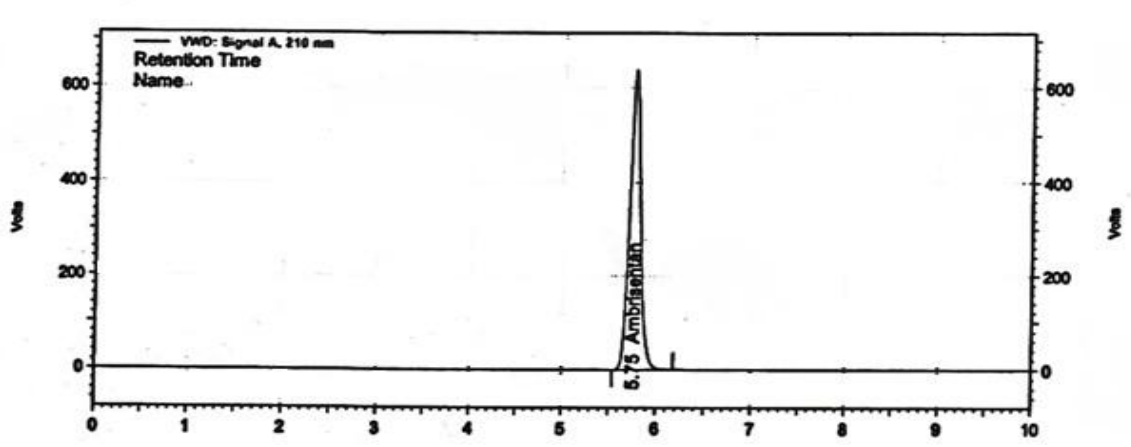


Figure10: HPLC chromatogram for fourth trial

Table: 12 Results for trial 4

Drug	Retention time	Tailing factor	Theoretical plates
Ambrisentan	5.75	2.25	7653

Discussion

The analyte peak was well eluted but the suitability parameter was not within the limits.

Trial No.5

Preparation of Buffer

2.72 gms of potassium dihydrogen orthophosphate is dissolved in 1000 ml of water and pH is adjusted to 3.±0.05 with dilute phosphoric acid, then sonicated and filtered through 0.45µ membrane filter.

Mobile phase preparation

The mobile phase was prepared by mixing mobile phase A: B in the ratio of 40:60 v/v.The mobile phase A is potassium dihydrogen orthophosphate buffer and mobile phase B is Acetonitrile: water (90:10v/v).Then sonicated and filtered through 0.45µ membrane filter.

Chromatographic conditions

Standard solution of Ambrisentan was loaded in the vial, injected and run for 10min. The HPLC parameters were set in the method as follows.

- Column : Kromosil C_{18}, 250×4.6 mm, 5µm
- Mobile phase : A:B(40:60 v/v)
  - Mobile phase A : Potassium dihydrogen orthophosphate buffer (pH 3.0)
  - Mobile phase B : Acetonitrile: water (90:10 v/v)
- Column temperature : Ambient
- Flow rate : 1mL/min
- Injection volume : 10µL
- Wave length : 210nm
- Run time : 10min

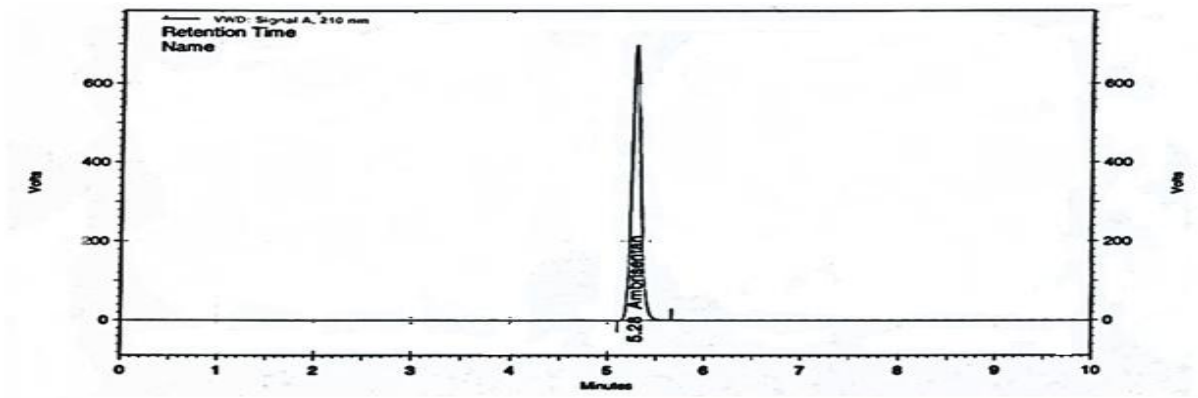


Figure 11: HPLC chromatogram for fifth trial

Table: 13 Results for trial 5

Drug	Retention time	Tailing factor	Theoretical plates
Ambrisentan	5.28	2.45	6509

Discussion

The elution of the analyte peak was satisfactory but the system suitability parameter was not within the limits.

Trial No.6

Analytical method development optimization

Preparation of Buffer

2.72 gms of potassium dihydrogen orthophosphate is dissolved in 1000 ml of water and pH is adjusted to 3.±0.05 with dilute phosphoric acid, then sonicated and filtered through 0.45µ membrane filter.

Mobile phase preparation

The mobile phase was prepared by mixing mobile phase A: B in the ratio of 30:70 v/v.The mobile phase A is potassium dihydrogen orthophosphate buffer and mobile phase B is Acetonitrile: water (90:10v/v), then sonicated and filtered through 0.45µ membrane filter.

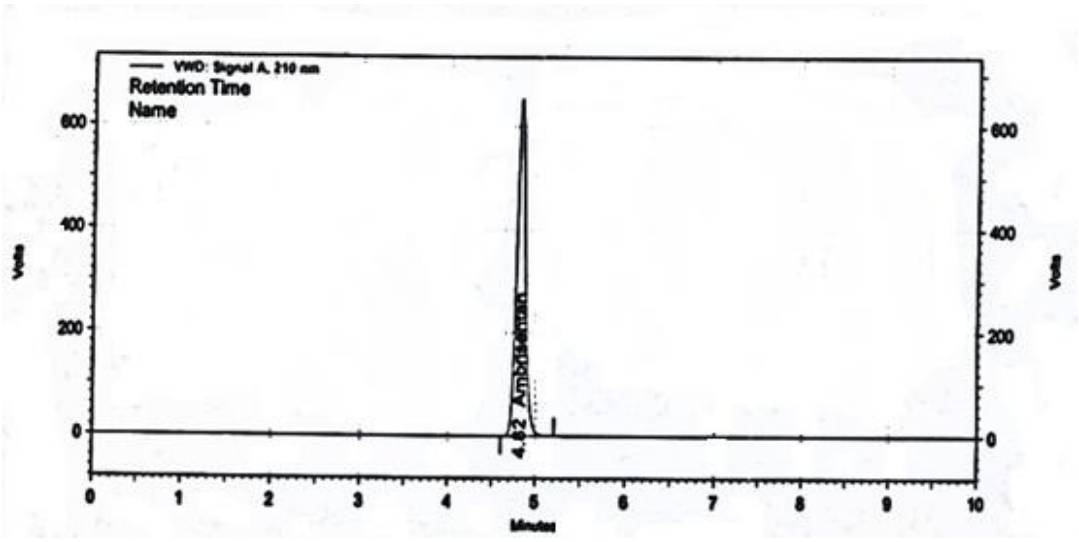


Figure 12: HPLC chromatogram for sixth trial

Table: 14 Results fortrial 6

Drug	Retention time	Tailing factor	Theoretical plates
Ambrisentan	4.82	0.85	11509

Discussion

The analyte peak was well eluted and all the system suitability parameters are within the acceptable limits. Hence this method is considers as optimized and validated as per ICH guidelines.

6.2 Optimized Analytical Method

So after six trials it has been observed that trial 6 was optimized method for carry out the validation of Ambrisentan in marketed tablet formulation. Chromatographic condition for optimized method are given below

Chromatographic conditions

Standard solution of Ambrisentan was loaded in the vial. The HPLC parameters were set in the method as per table 8, injected and run for 10 min.

Table: 8 Chromatographic Conditions

PARAMETERS	DESCRIPTION
Column	KromosilC$_{18}$, 250×4.6 mm, 5µm
Temperature	35^{0}C
Mobile phase	A:B(30:70)
Mobile phase A	Potassium dihydrogen orthophosphate buffer of pH 3.0±0.05
Mobile phase B	ACN:Water (90:10)
Flow Rate	1.0 ml/min
Injection Volume	10µL
Detector wave length	210nm
Run time	10 min

6.3 Assay

Procedure of the preparation of standard solution & sample solution

6.3.1 Preparation of working standard solution

Approximately 25mg of Ambrisentan (WS) was weighed and transferred into50mL volumetric flask, diluted to 30 ml with mobile phase and sonicated for 15minutes. Then the volume was made up to 50mL with mobile phase and filtered through 0.45µ membrane filter. Further 5ml of above solution was diluted to 25 ml and mixed to get a concentration of 100 µg/ml.

6.3.2 Preparation of sample solutions

20 tablets were powdered and weigh and transfer tablet powder equivalent to 50 mg of Ambrisentan into 50 ml volumetric flask, diluted to 30 ml with mobile phase and sonicated for 15 mins and diluted to volume and filtered through 0.45µ membrane filter. Further dilute 5 ml of this solution to 25 ml with mobile phase and mixed to get a concentration of 100 µg/ml.

Calculation:

The amount of Ambrisentan present in marketed formulation each tablet was calculated by using the following formula:

Amount present

$$= \frac{\text{Sample Area}}{\text{Standard Area}} \text{ X } \frac{\text{Standard weight}}{\text{Standard Dilution}} \text{ X } \frac{\text{Sample Dilution}}{\text{Sample weight}} \text{X} \frac{\text{WS purity}}{100} \text{ X Average weight}$$

$$\textbf{Assay} = \frac{\text{Amount present}}{\text{Label Claim}} \text{ X } 100$$

Average weight of 20 tablets = 91.6 mg.

Weight taken = 461.2 mg.

Label claim: 5 mg of Ambrisentan.

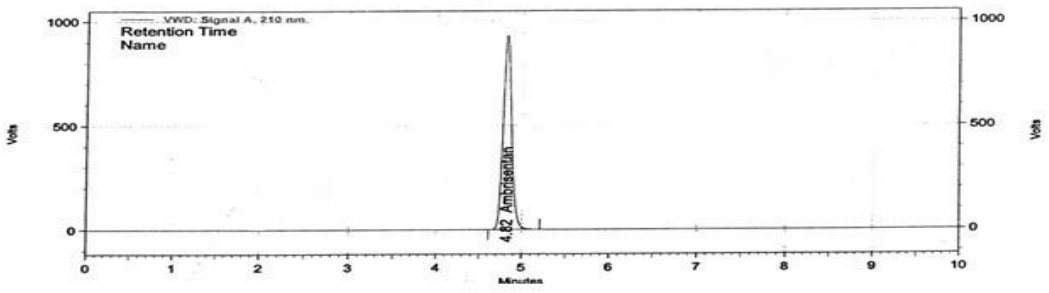


Figure 13: HPLC chromatogram of Ambrisentan for marketed formulation

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	4.82	74991596	11087	0.91

Table.9 Tabular Column for the Assay of Marketed formulation

S. No	Weight taken	Sample area	Standard area	Content/Tablet in mg	Percentage purity
1	461.2 mg	74991596	75174299	5.01	100.25

Discussion

The percentage purity of Ambrisentan in tablet dosage form was calculated. The percentage purity was found of Ambrisentan was found to be 100.25

7. VALIDATION OF DEVELOPED METHOD

7.1 PREPARATION OF SOLUTION

7.1.1 Preparation of Phosphate Buffer Solution

2.72 gms of potassium dihydrogen orthophosphate is dissolved in 1000 ml of water and pH is adjusted to 3.±0.05 with dilute phosphoric acid, then sonicated and filtered through 0.45µ membrane filter.

7.1.2 Preparation of mobile phase

The mobile phase was prepared by mixing mobile phase A: B in the ratio of 30:70 v/v.The mobile phase A is potassium dihydrogen orthophosphate buffer and mobile phase B is Acetonitrile: water (90:10v/v), then sonicated and filtered through 0.45µ membrane filter

7.1.3 Preparation of working standard solution

Approximately 25mg of Ambrisentan (WS) was weighed and transferred into50mL volumetric flask, diluted to 30 ml with mobile phase and sonicated for 15minutes. Then the volume was made up to 50mL with mobile phase and filtered through 0.45µ membrane filter. Further 5ml of above solution was diluted to 25 ml and mixed to get a concentration of 100 µg/ml.

7.1.4 Preparation of sample solutions

20 tablets were powdered and weigh and transfer tablet powder equivalent to 50 mg of Ambrisentan into 50 ml volumetric flask, diluted to 30 ml with mobile phase and sonicated for 15 mins and diluted to volume and filtered through 0.45µ membrane filter. Further dilute 5 ml of this solution to 25 ml with mobile phase and mixed to get a concentration of 100 µg/ml.

The amount of Ambrisentan present in each tablet was calculated by using the following formula:

$$\text{Amount present} = \frac{\text{Sample Area}}{\text{Standard Area}} \text{ X } \frac{\text{Standard weight}}{\text{Standard Dilution}} \text{ X } \frac{\text{Sample Dilution}}{\text{Sample weight}} \text{X} \frac{\text{Percentage purity}}{100} \text{ X Average weight}$$

$$\% \text{ Amount present} = \frac{\text{Amount present}}{\text{Label Claim}} \text{ X } 100$$

7.2 ANALYTICAL METHOD VALIDATION

The developed method was validated according to ICH guidelines

Method validation of Ambrisentan

The mobile phase was prepared and all parameters were set as per the above optimized method.

7.2.1 Specificity

Ambrisentan Identification

Solutions of standard and Sample were prepared as per test method and injected into the chromatographic system.

Acceptance criteria

Chromatogram of Standard and sample should be identical with near/same retention time.

Blank interference

A study to establish the interference, blank detection was conducted. Mobile phase was injected as per the test method.

Acceptance criteria

Chromatogram of blank did not show any peak at the retention time of analyte peak.

The blank, placebo, standard and sample solutions were injected into the chromatograph and the chromatograms ware recorded and presented below.

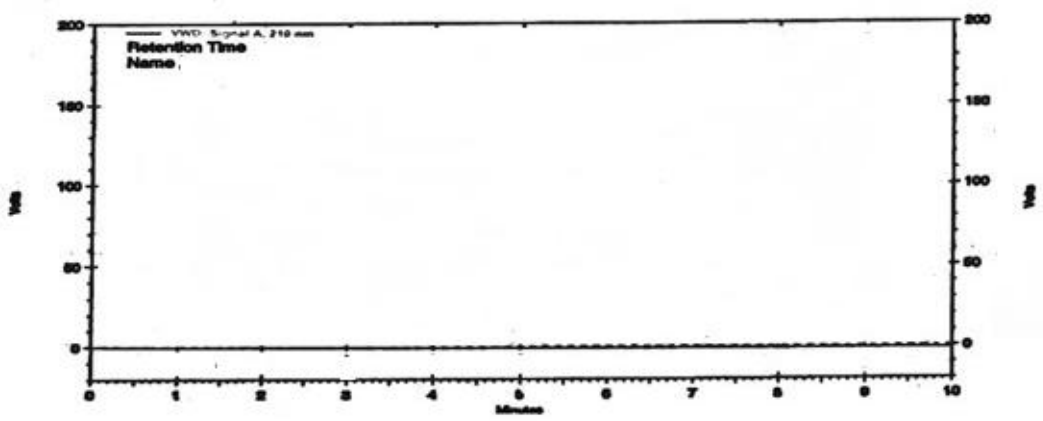


Figure 14: HPLC Chromatogram of blank (mobile phase)

Drug	Retention time	Tailing factor	Theoretical plates
Ambrisentan	------	------	------

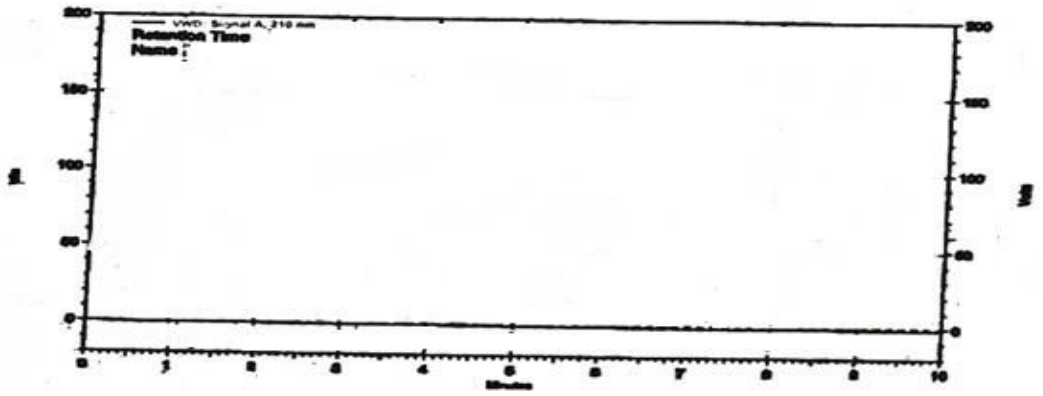


Figure 15: HPLC chromatogram of Placebo

Drug	Retention time	Tailing factor	Theoretical plates
Ambrisentan	------	------	------

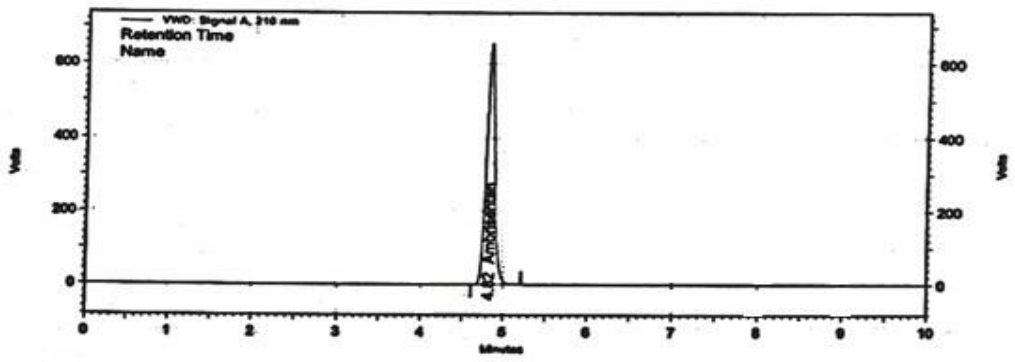


Figure 16: HPLC chromatogram of standard Ambrisentan

Drug	Retention time	Tailing factor	Theoretical plates
Ambrisentan	4.82	0.85	11509

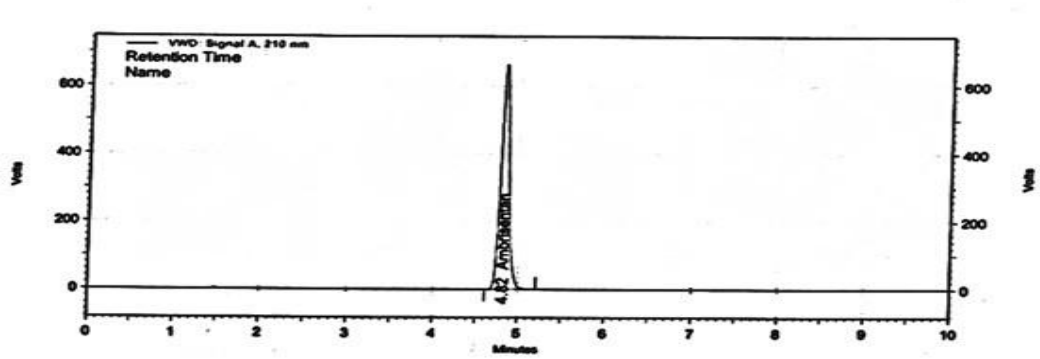


Figure 17: HPLC chromatogram of sample

Drug	Retention time	Tailing factor	Theoretical plates
Ambrisentan	4.82	0.81	11091

Observation : No peaks were found at retention time 4.82 min

Discussion : As no peaks were found at retention time of 4.82 min the proposed method was specific for the detection of Ambrisentan.

7.2.2 Linearity and Range

The linearity of calibration curves in standard solution was checked over the concentration ranges of about 50-140 µg/ml for Ambrisentan. The total eluting time was less than 5min. The linearity plot is shown in fig 17.

The peak area and concentration were plotted to get a standard calibration curve. The correlation coefficient and regression coefficient were calculated.

Linearity 50%

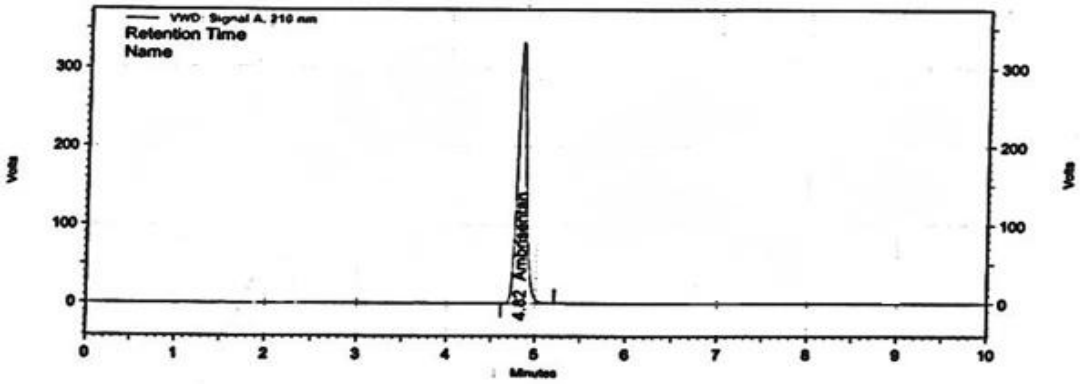


Figure 18: HPLC chromatogram of Ambrisentan for linearity 50%.

S.No	Conc.(µg/ml)	Name	RT	Area	Platecount	Tailing
1	50	Ambrisentan	4.82	38232397	10930	0.85

Linearity 60%

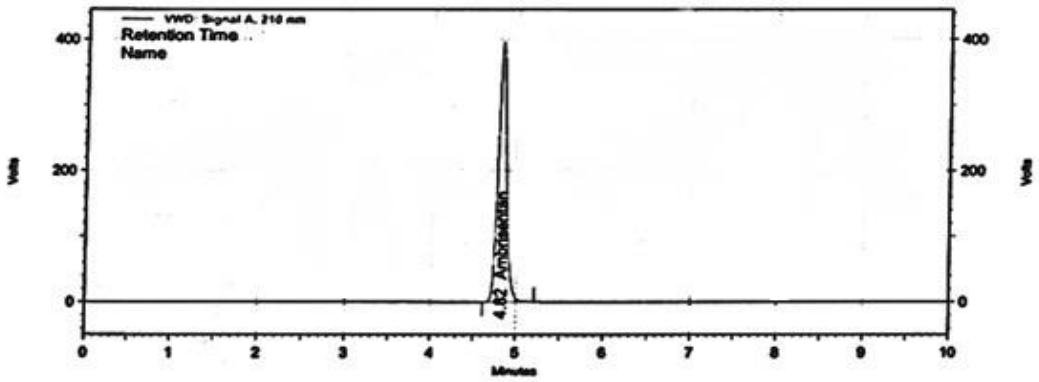


Figure 19: HPLC chromatogram of Ambrisentan for linearity 60%.

S. No	Conc(µg/ml)	Name	RT	Area	Platecount	Tailing
1	60	Ambrisentan	4.82	45520461	11564	0.93

Linearity 80%

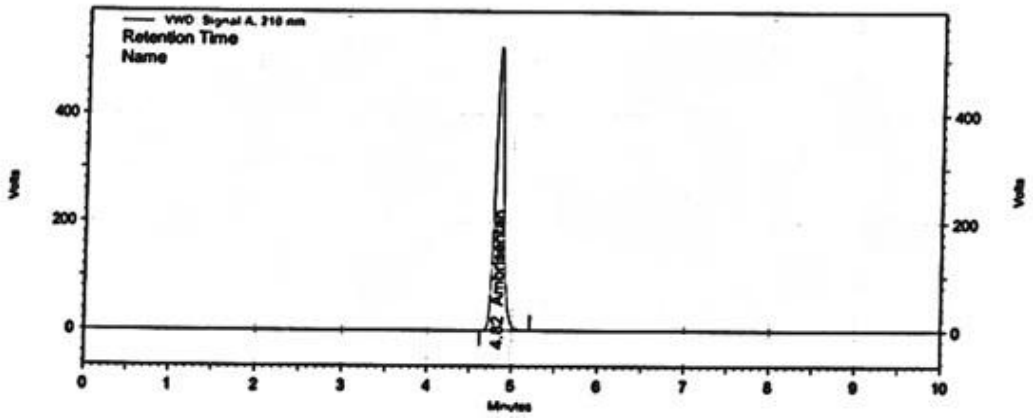


Figure 20: HPLC chromatogram of Ambrisentan for linearity 80%.

S.No	Conc.(µg/ml)	Name	RT	Area	Platecount	Tailing
1	80	Ambrisentan	4.82	60179623	11512	0.97

Linearity 100%

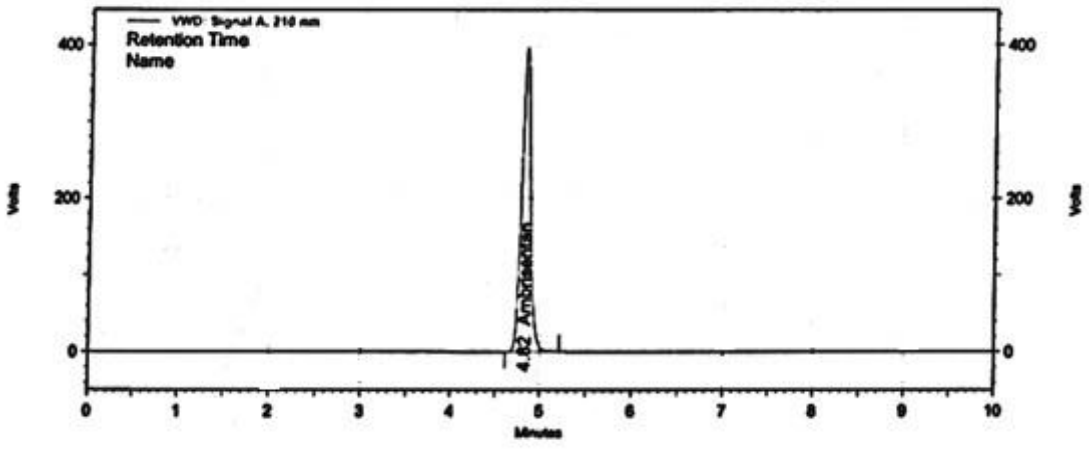


Figure 21: HPLC chromatogram of Ambrisentan linearity 100 %.

S.No	Conc.(µg/ml)	Name	RT	Area	Platecount	Tailing
1	100	Ambrisentan	4.82	76181304	11465	0.95

Linearity 120%

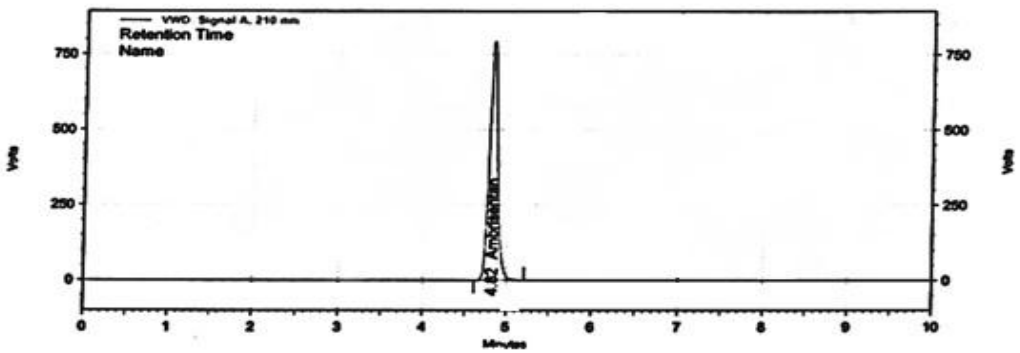


Figure 22: HPLC chromatogram of Ambrisentan linearity 120 %

S.No	Conc.(µg/ml)	Name	RT	Area	Platecount	Tailing
1	120	Ambrisentan	4.82	91097774	11504	0.83

Linearity 150%

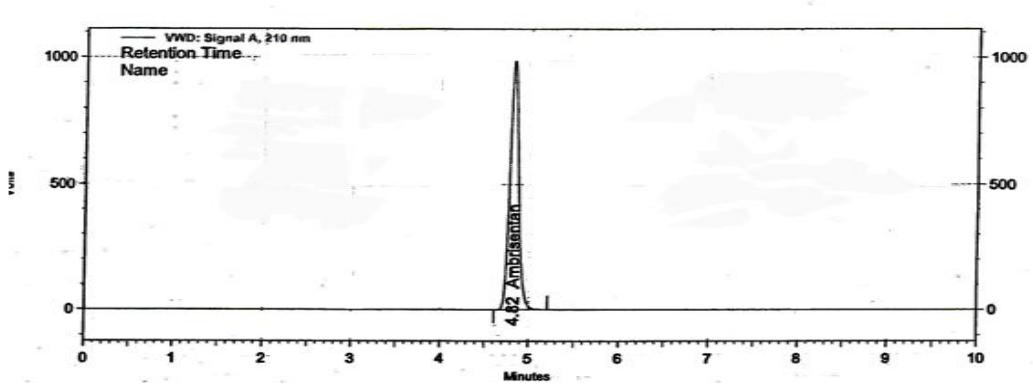


Figure 23: HPLC chromatogram of Ambrisentan linearity 150 %

S.No	Conc.(µg/ml	Name	RT	Area	Platecount	Tailing
1	150	Ambrisentan	4.82	107102232	11697	0.92

Figure 24: Linearity plot of Ambrisentan

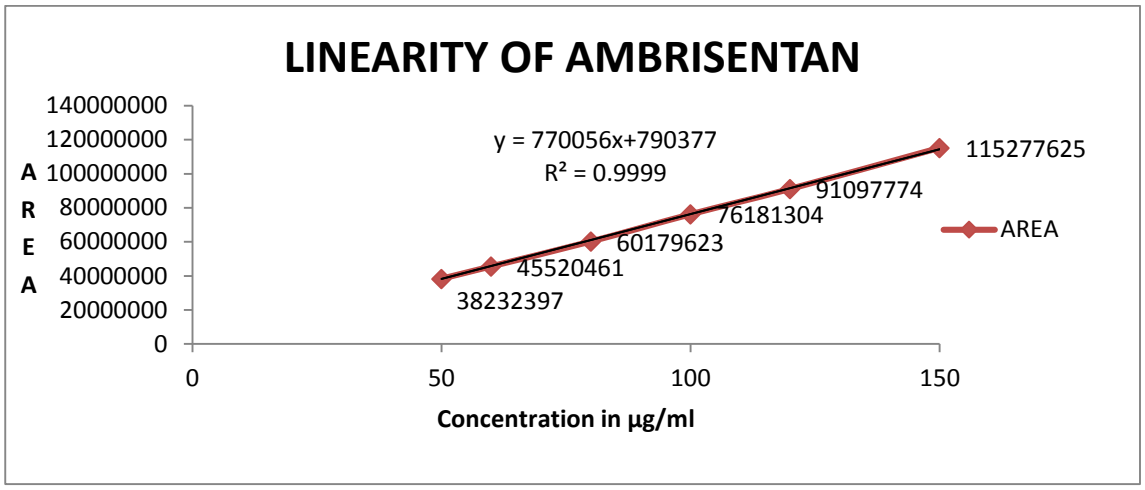


Table: 10 Linearity of Ambrisentan

S .NO	CONC. (%)	AREA
1.	50	38232397
2.	60	45520461
3.	80	60179623
4.	100	76181304
5.	120	91097774
6.	150	115277625

Acceptance criteria: Correlation coefficient ≥ 0.997

Discussion The linearity of the method was demonstrated over the concentration range of 50-150 µg/ml for Ambrisentan. The calibration curve for Ambrisentan was plotted. The correlation coefficient for Ambrisentan was found to be 0.999.

7.2.3 Accuracy

Accuracy of the method was determined by recovery experiments. Recovery study can be carried out by spiking study or swab study (usually for cleaning validation). In my experiment I performed by spiking method. To the formulation, the reference standards of

the drug were added at the level of 50%, 100%, 150%. The recovery studies were carried out by triplicate injection and the percentage recovery and percentage relative standard deviation were calculated and shown in table 17.

Accuracy 50% injection 1

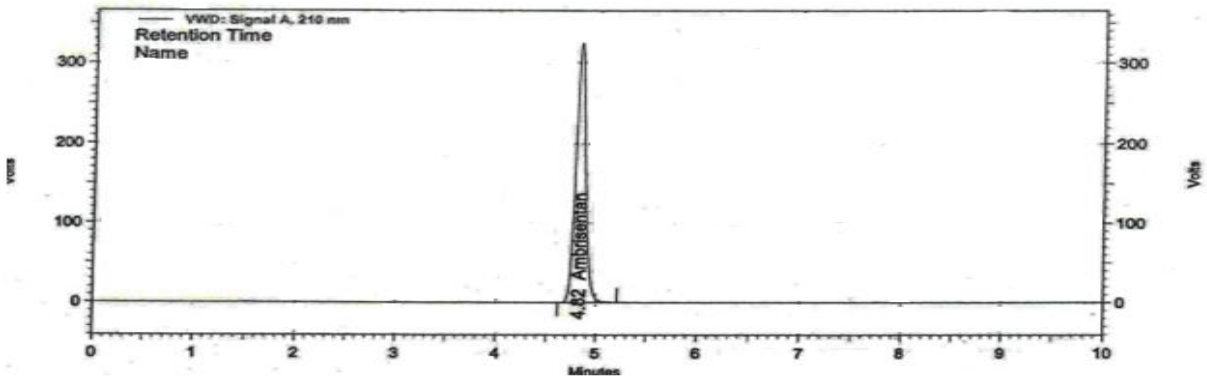


Figure 24: HPLC chromatogram of Ambrisentan for Accuracy 50% Inj (1)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	37229585	11017	0.96

Accuracy 50% injection 2

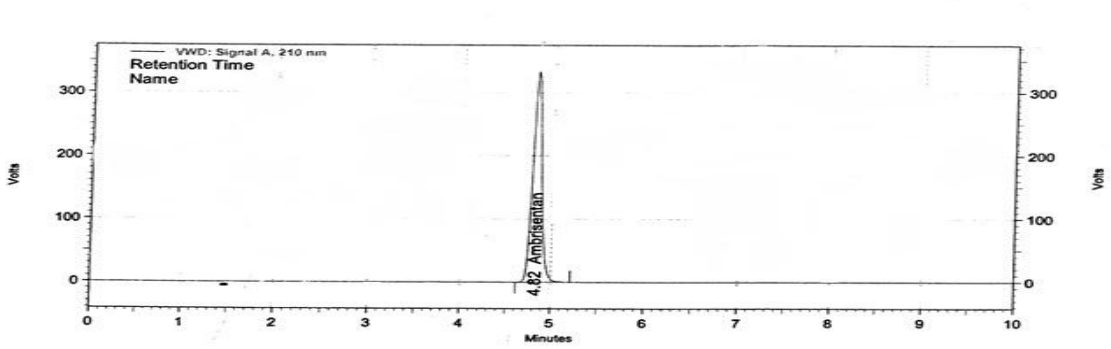


Figure 25: HPLC chromatogram of Ambrisentan for Accuracy 50% Inj (2)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	37532397	10978	0.81

Accuracy 50% injection 3

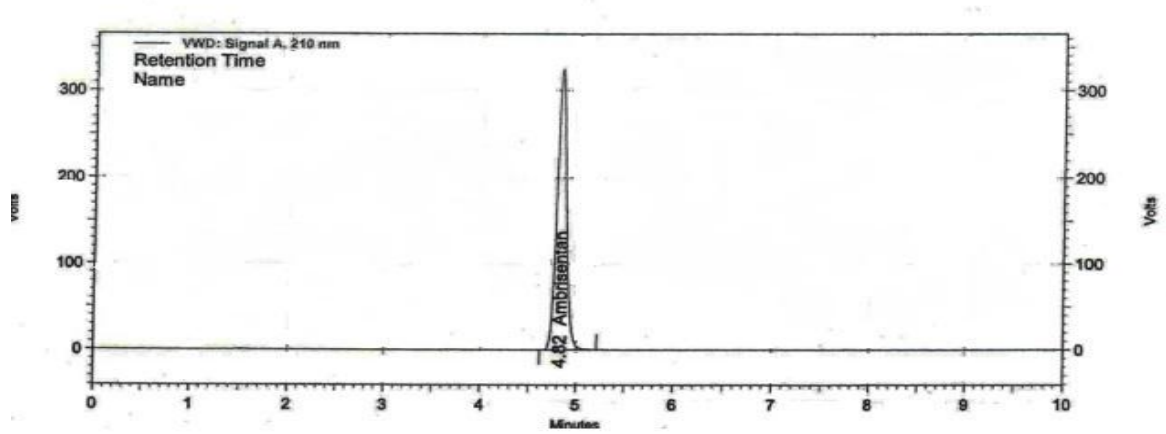


Figure 26: HPLC chromatogram of Ambrisentan for Accuracy 50% Inj (3)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	37235987	11126	0.97

Accuracy 100% injection 1

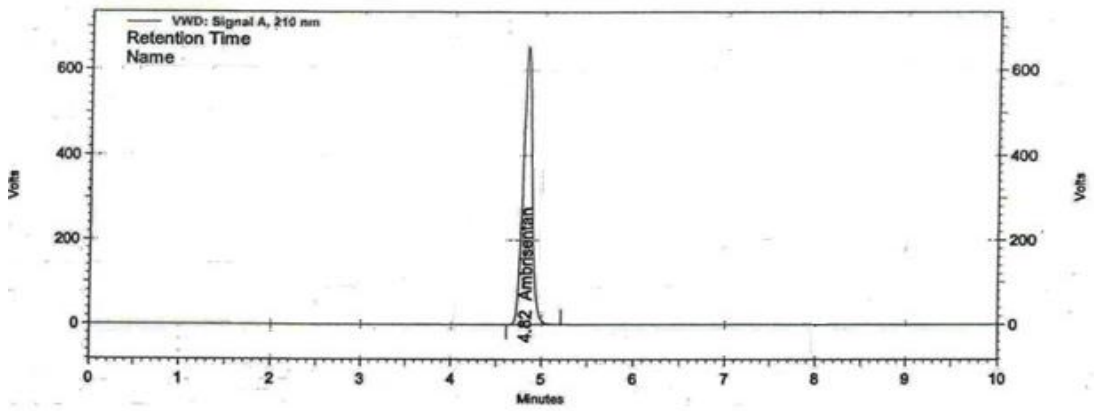


Figure 27: HPLC chromatogram of Ambrisentan for Accuracy 100% Inj (1)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	75491039	11413	0.89

Accuracy 100% injection 2

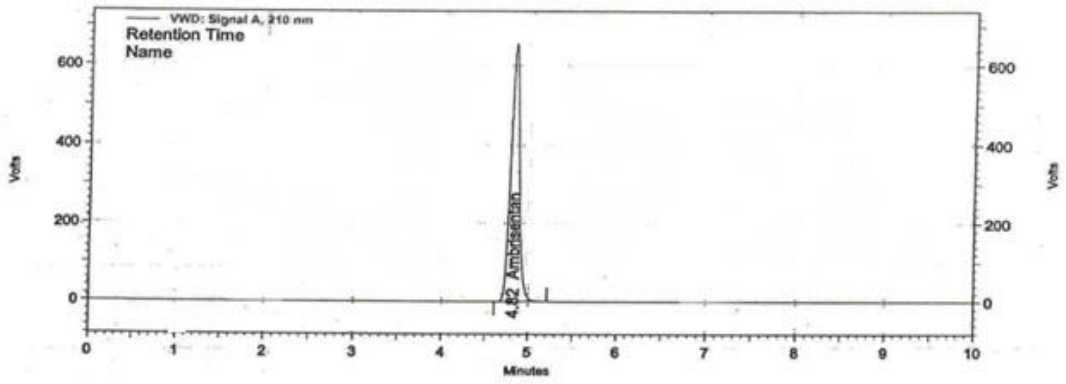


Figure 28: HPLC chromatogram of Ambrisentan for Accuracy 100% Inj (2)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	76181304	11604	0.83

Accuracy 100% injection 3

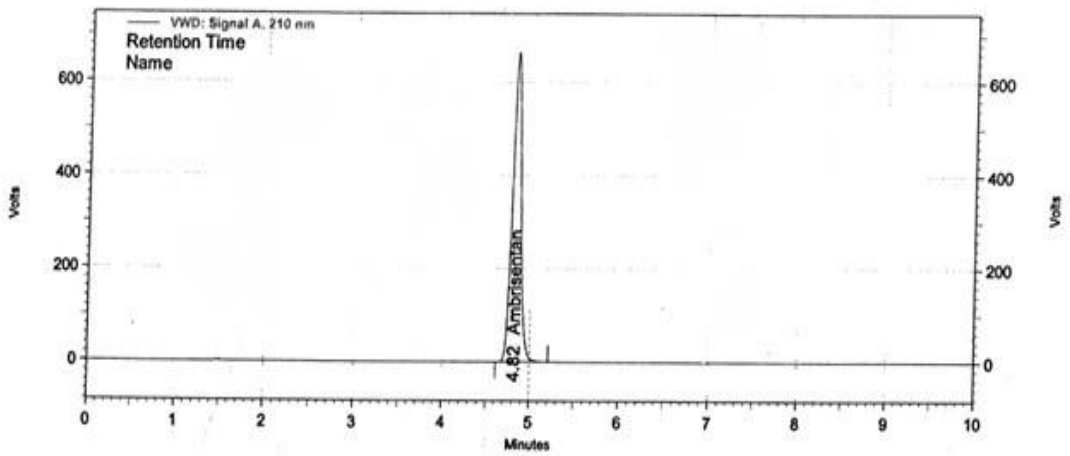


Figure 29: HPLC chromatogram of Ambrisentan for Accuracy 100% Inj (3)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	75914302	11369	0.79

Accuracy 150% injection 1

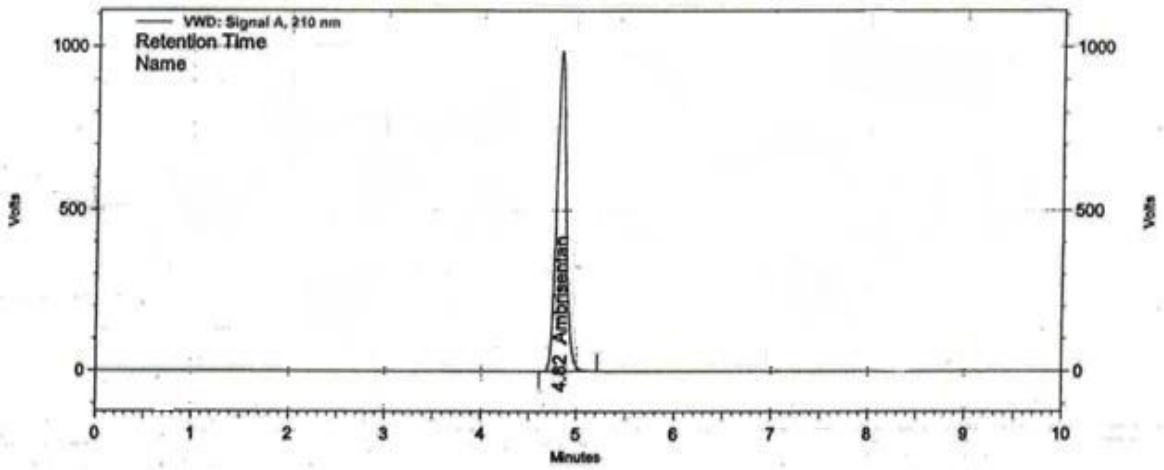


Figure 30: HPLC chromatogram of Ambrisentan for Accuracy 150% Inj (1)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	114088905	12239	0.85

Accuracy 150% injection 2

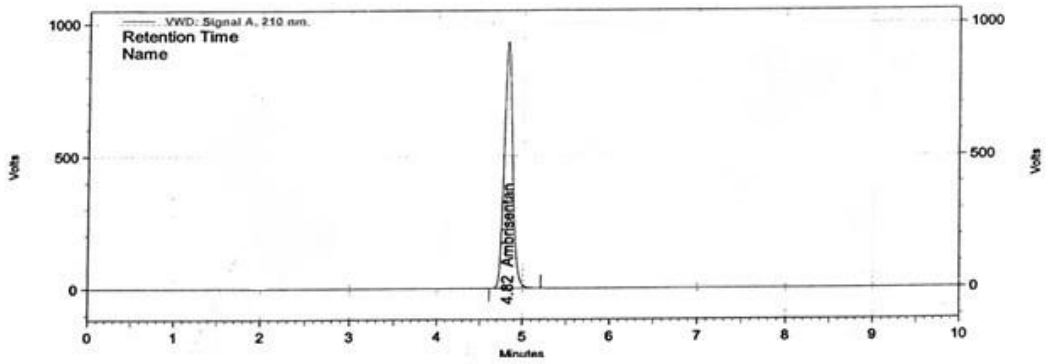


Figure 31: HPLC chromatogram of Ambrisentan for Accuracy 150% Inj (2)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	114088905	12119	0.86

Accuracy 150% injection 3

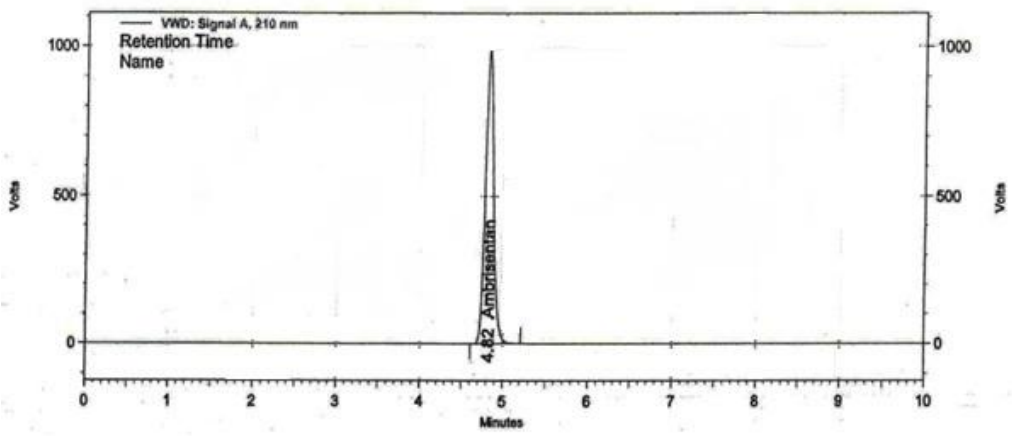


Figure 32: HPLC chromatogram of Ambrisentan for Accuracy 150% Inj (3)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	115277625	12089	0.83

Table: 11 Accuracy results for Ambrisentan

Spiked Level	Peak Area	Amount Found In mcg/ml	% Recovery	%Mean Recovery	% RSD
50%	37229585	50.10	99.22		
50%	37532397	50.45	99.37	99	0.46
50%	37235987	50.11	99.02		
100%	75491039	101.60	100.26		
100%	76181304	102.53	100.74	100	0.45
100%	75914302	102.17	99.95		
150%	114088905	153.55	100.25		
150%	115277625	155.15	101.59	101	0.53
150%	114971209	154.74	101.61		

The % recovery of the sample was calculated by the following formula--

$$\% \text{ Recovery} = \frac{\text{Actual calculated amount present in recovery sample}}{\text{Amount spiked in to the recovery sample} + \text{Calculated theoritical preexisting amount}} \text{ X } 100$$

Acceptance Criteria

The percentage recovery calculated should be between 98 to 102%. And % RSD of each level should not less than 2.0

Discussion

The percentage recovery of Ambrisentan was found 99%, 100% and 101% for accuracy 50%, 100% and 150% samples respectively. The percentage RSD of the samples was also found less than 2.

7.2.4 Precision

Demonstrate the method precision by preparing six samples as per the test method of a single batch representing the 100% of test concentration. Determine the assay of these samples and evaluate the precision of the method by the % RSD of the assay results. The results were mentioned in table18.

Precision injection 1

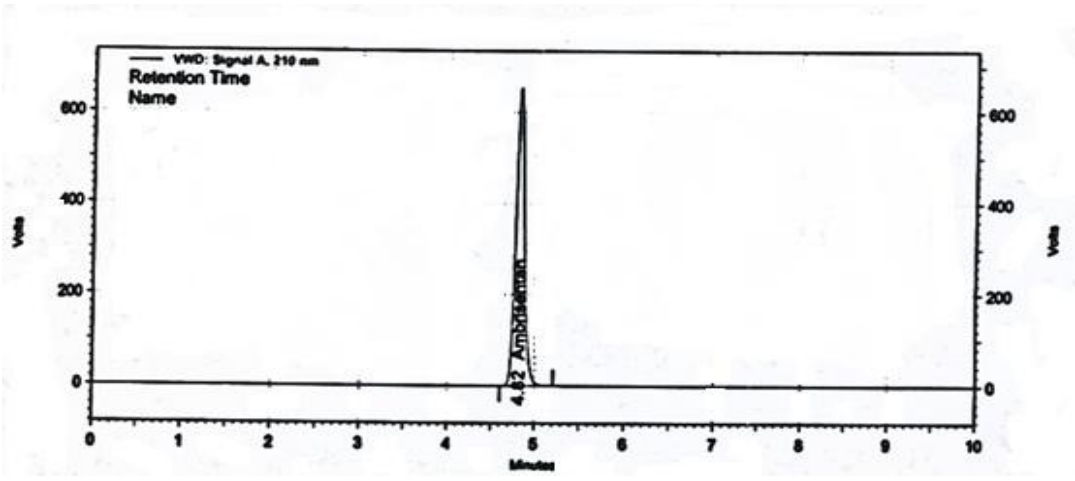


Figure33: HPLC chromatogram of Ambrisentan for Precision Inj (1)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	74922610	11491	0.84

Precision injection 2

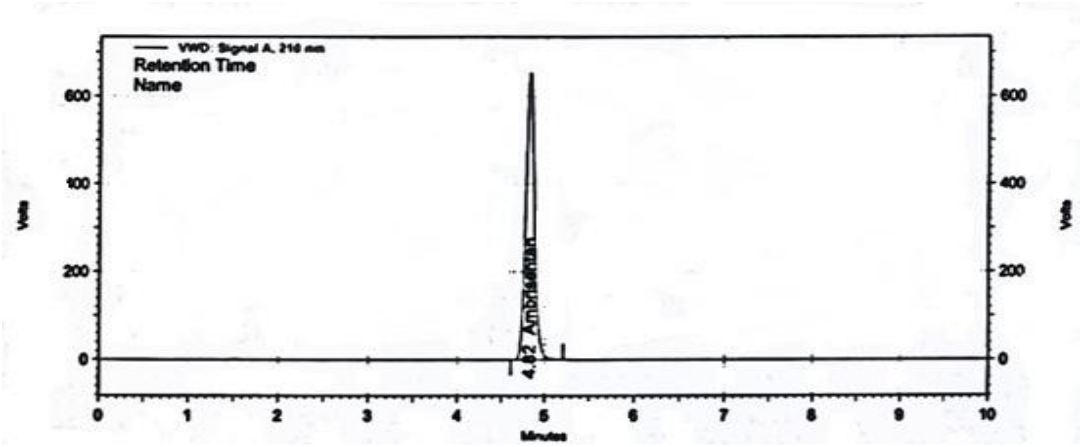


Figure 34: HPLC chromatogram of Ambrisentan for Precision Inj (2)

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	4.82	75155992	11097	0.93

Precision injection 3

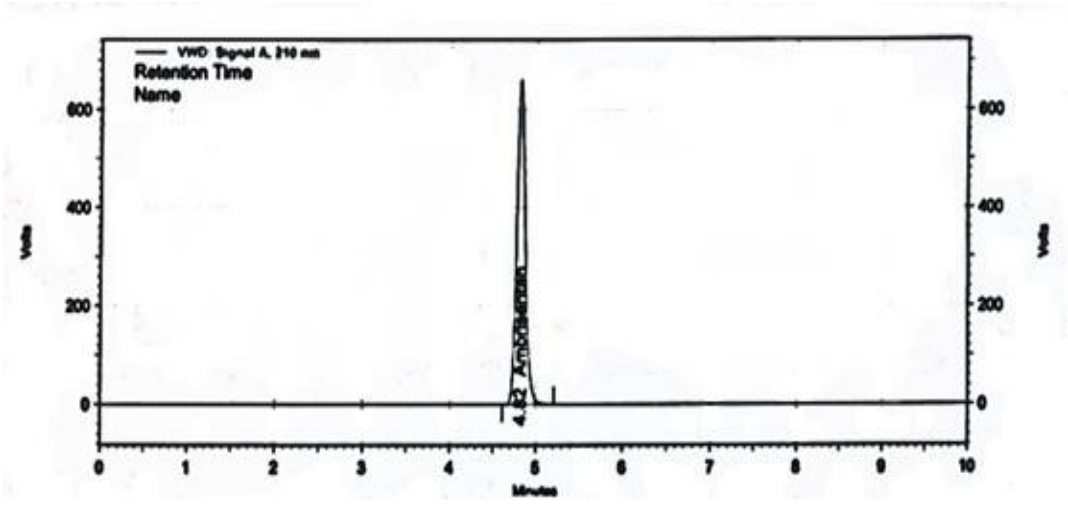


Figure 35: HPLC chromatogram of Ambrisentan for Precision Inj (3)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.82	75403775	11219	0.87

Precision injection 4

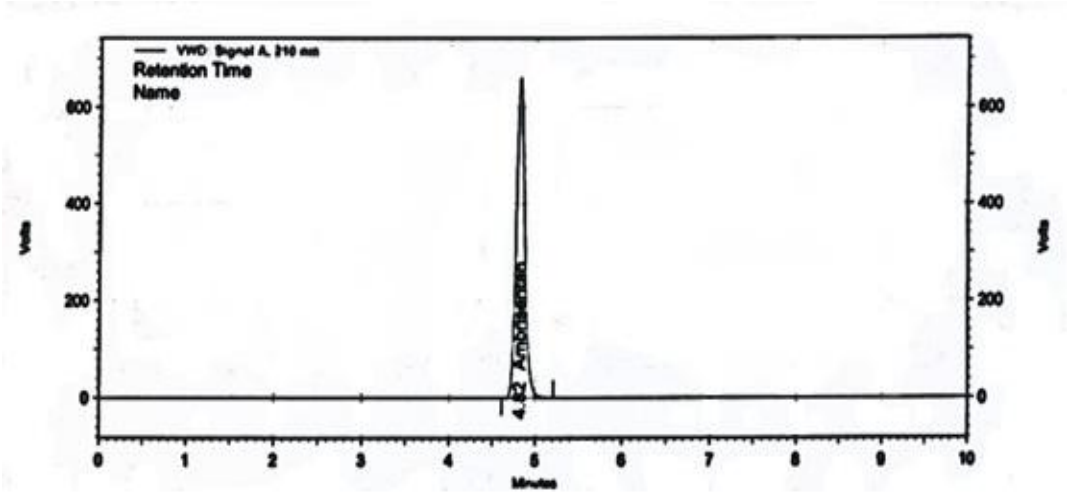


Figure36: HPLC chromatogram of Ambrisentan for Precision Inj (4)

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	4.82	75169587	11157	0.91

Precision injection 5

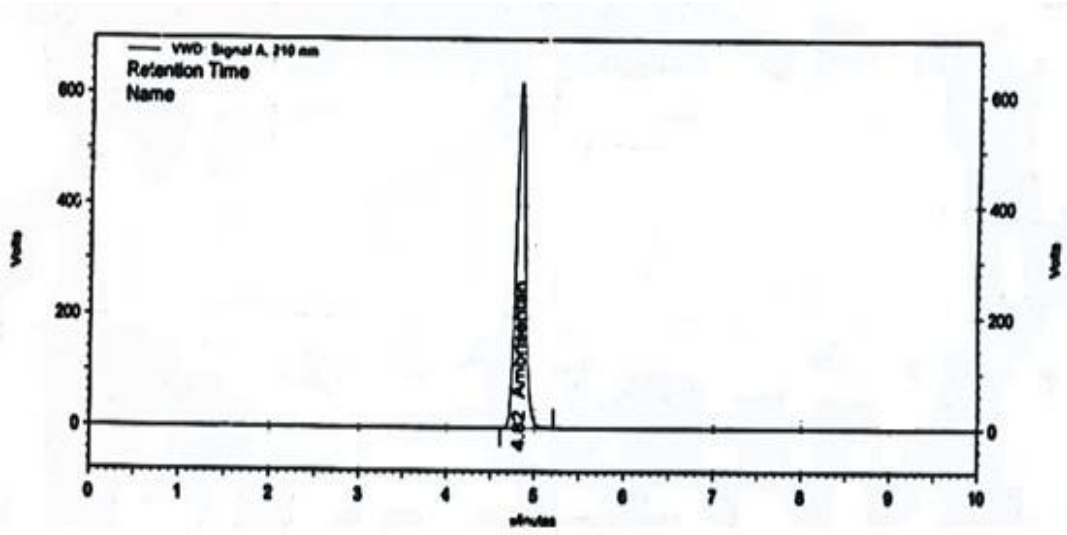


Figure 37: HPLC chromatogram of Ambrisentan for Precision Inj (5)

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	4.82	75219532	11329	0.87

Precision injection 6

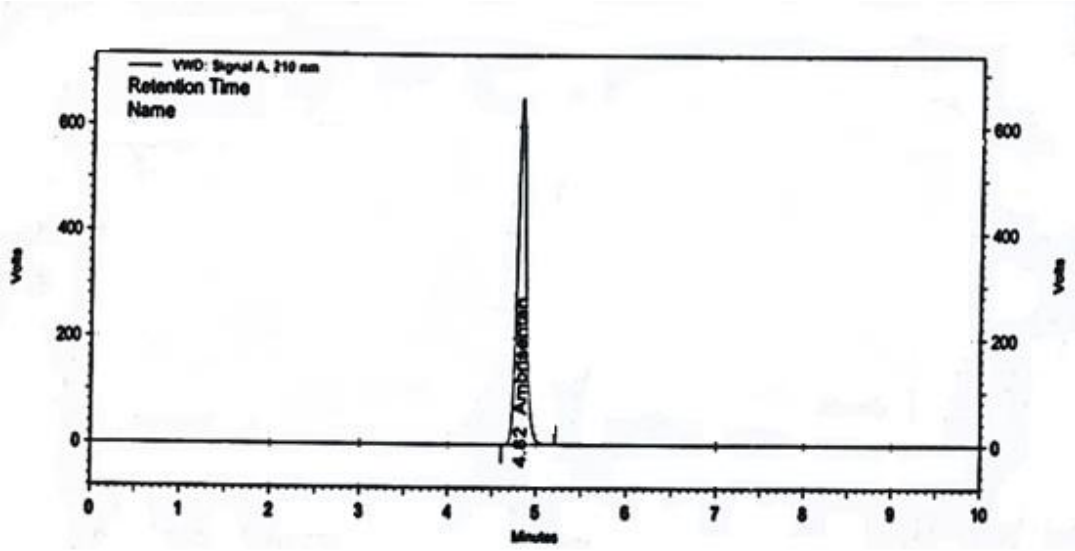


Figure 38: HPLC chromatogram of Ambrisentan for Precision Inj (6)

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	4.82	75091152	11279	0.94

Table: 12 Precision results for Ambrisentan

Drug Concentration	Peak Area	Retention Time
Ambrisentan (100 µg/ml)	74922610	4.82
	75155992	4.82
	75403775	4.82
	75169587	4.82
	75219532	4.82
	75091152	4.82
Standard Deviation	157588	NIL
%RSD	0.45	NIL

Acceptance criteria: Percentage RSD ≤ 2

Discussion

The above precision study was assessed by repeatability tests. The % RSD for the area of Ambrisentan was found to be 0.45, which was well within the acceptance criteria limit.

7.2.5 Limit of Detection & Limit of Quantitaton

The LOD and LOQ of ESC and ETZ shall be estimated from the standard deviation of the response and the slope of the calibration curve by using the following formula

$$\text{LOD} = \frac{3.3\times\sigma}{S} \qquad \text{LOQ} = \frac{10\times\sigma}{S}$$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

Table 13: LOD & LOQ DETERMINATION

Name	Slope	Standard deviation of response	LOD (µg/ml)	LOQ (µg/ml)
Ambrisentan	770056	562962.7	4.51	13.68

Report: The LOD & LOQ of Ambrisentan for this method were found to be 4.51 µg/ml, and 13.68 µg/ml respectively.

7.2.6 Robustness

Robustness of the method was checked by small deliberate changes in the method parameters during the normal use. For this study flow rate, mobile phase composition, pH and temperature parameters were changed. The results were mentioned in table 19.

Robustness ((Flow 0.9ml/min)

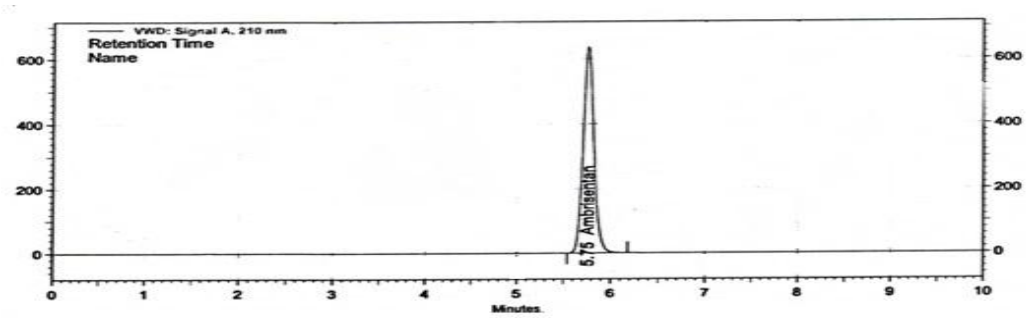


Figure39: HPLC chromatogram of Ambrisentan for robustness (Flow 0.9ml/min)

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	5.75	80823412	12239	1.18

Robustness (Flow 1.1 ml/min)

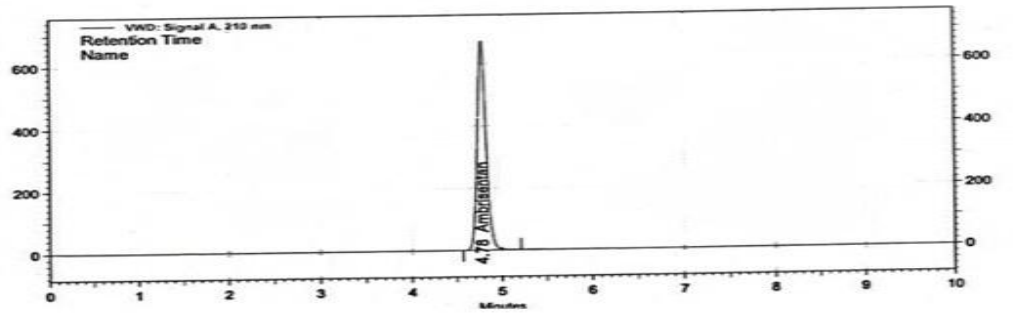


Figure 40: HPLC chromatogram of Ambrisentan for robustness (Flow 1.1 ml/min)

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	4.78	79878490	10930	1.23

Table 14: Result of flow rate change

S.No	Flow Rate	Retention Time	Area
1.	**0.9 ml/minute**	**5.75**	**80823412**
2.	**1 ml/minute**	**4.82**	**74991596**
3.	**1.1 ml/minute**	**4.78**	**79878490**

Robustness (Temp 30˚C)

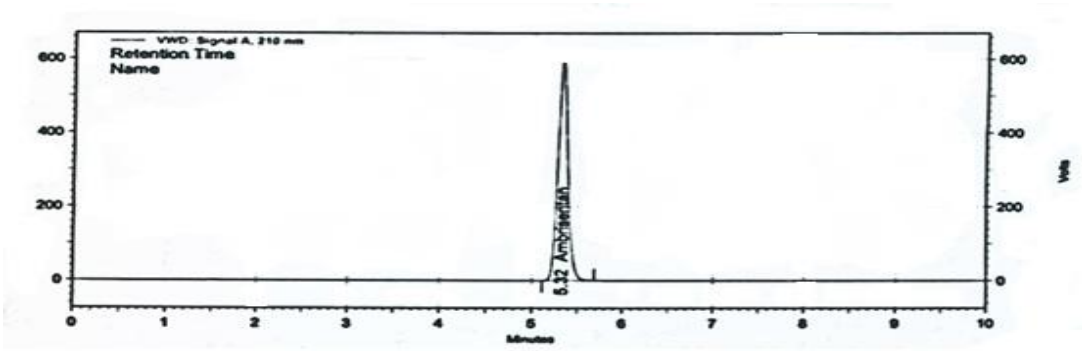


Figure 41: HPLC chromatogram of Ambrisentan for robustness (Temp 30˚C)

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	5.32	76014087	11432	1.18

Robustness (Temp 40˚C)

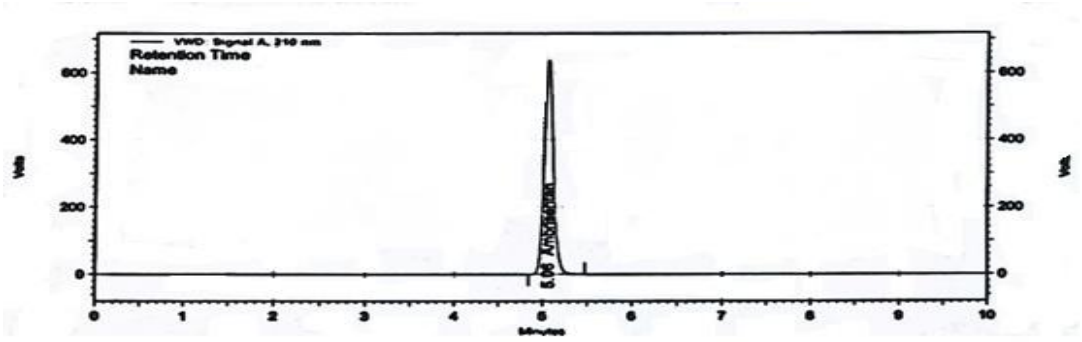


Figure 42: HPLC chromatogram of Ambrisentan for robustness (Temp 40˚C)

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	5.06	76292754	11629	1.18

Table 15: Result of Column temperature change

S.No	Column temp.	Retention Time	Area
1.	**30˚C**	**5.32**	**76014087**
2.	**Ambient**	**4.82**	**74991596**
3.	**40˚C**	**5.06**	**76292754**

Robustness (pH 2.8)

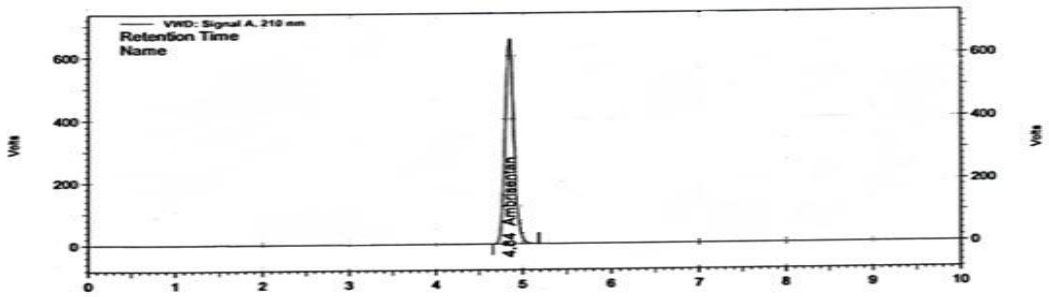


Figure 43: HPLC chromatogram of Ambrisentan for robustness (pH2.8)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.84	76275913	11089	1.21

Robustness (pH 3.2)

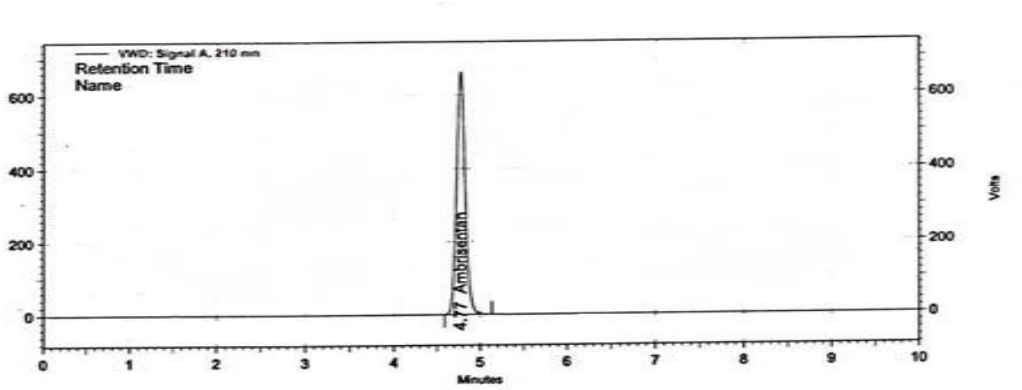


Figure 44: HPLC chromatogram of Ambrisentan for robustness (pH 3.2)

S. No	Name	RT	Area	Platecount	Tailing
1	Ambrisentan	4.77	76656284	10966	1.27

Table 16: Result of pH change

S.No	pH change	Retention Time	Area
1.	pH 2.8	4.84	76275913
2.	pH 2.8	4.82	74991596
3.	pH 2.8	4.77	76656284

Robustness (M.P Variation -2%)

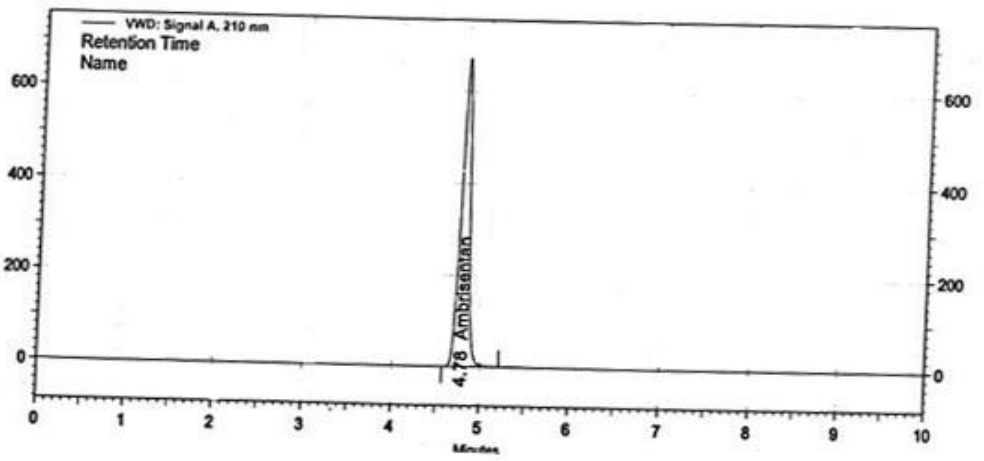


Figure 45: HPLC chromatogram of Ambrisentan for robustness (M.P Variation -2%)

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	4.78	77025377	11380	1.16

Robustness (M.P variation +2%)

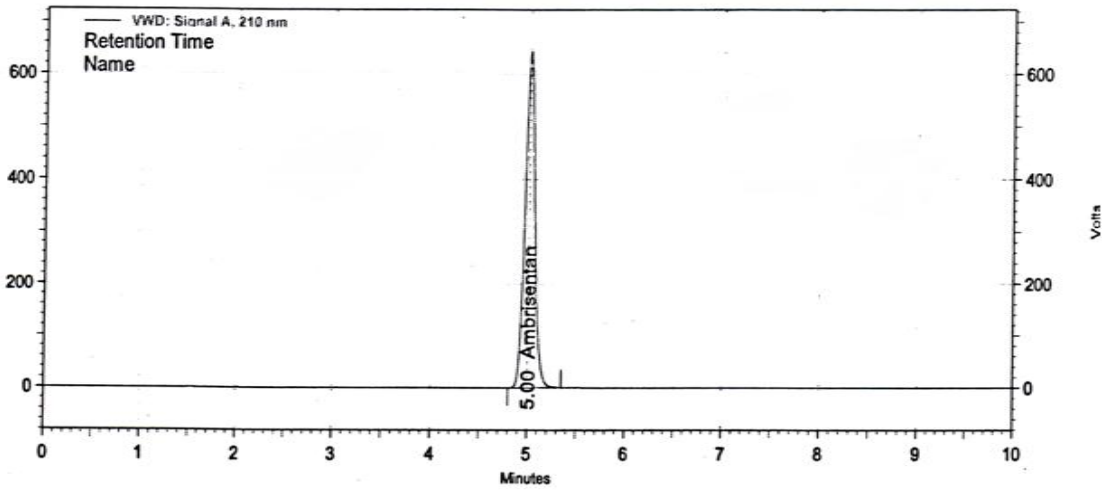


Figure 46: HPLC chromatogram of Ambrisentan for robustness (M.P variation +2%)

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	5.00	76656284	10966	1.27

Discussion:

The robustness was tested by changing the flow, temperature, pH and mobile phase composition in the chromatographic parameters. The %RSD was found to be 0.83, 0.25, 0.35, and 0.34 for flow, temperature, pH and mobile phase composition variation respectively.

7.2.7 System Suitability

Accurately 25mg of Ambrisentan was weighed and transferred into 50mL volumetric flask, diluted to 30 ml with mobile phase and sonicated for 15minutes. Then the volume was made up to 50mL and filtered through 0.45µ membrane filter. Further 5mL of above solution was diluted to 25 ml with mobile phase and mixed to get a concentration of 100 µg/ml. This Standard solution was injected in six replicate injections and the suitability parameters were calculated and given in table 20.

This parameter was tested by giving six replicate injections of the sample to check the system suitability parameters like asymmetry, theoretical plates, tailing factor etc.

Table.17 System suitability data of Ambrisentan

Parameter	Ambrisentan	Acceptance criteria
Theoretical plates	11916	>2000
Tailing factor	0.84	<2
Asymmetric factor	1.17	0.9-1.2

Discussion

The all system suitability parameters were within limits. The retention time for Ambrisentan was found to be 4.82 min.

7.2.8 Solution stability

This parameter was performed to check the stability of the solution to give unaffected results over the course of time. Solution stability was performed after 24 and 48 hours

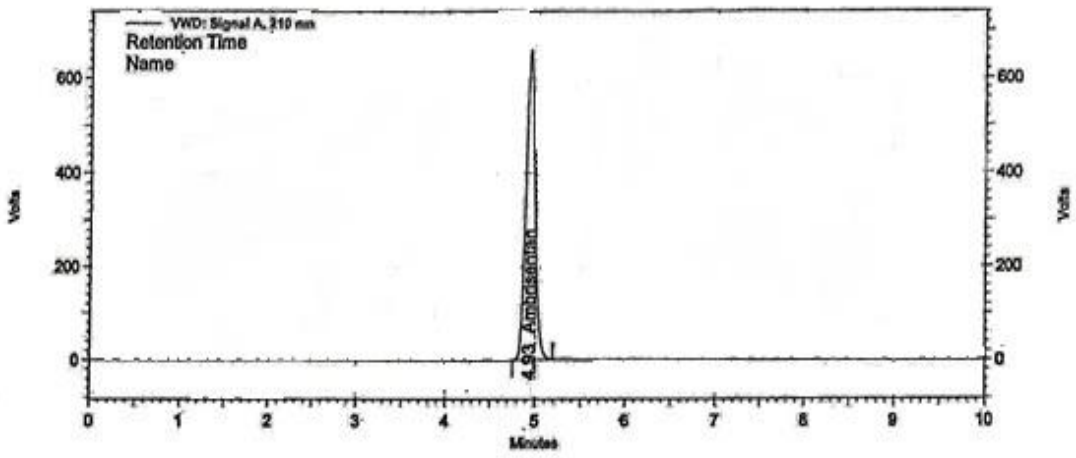


Figure 47: HPLC chromatogram of Ambrisentan for solution stability after 24 hrs

S. No	Name	RT	Area	Plate count	Tailing	Percentage purity
1	Ambrisentan	4.93	75550735	11765	1.25	100.34

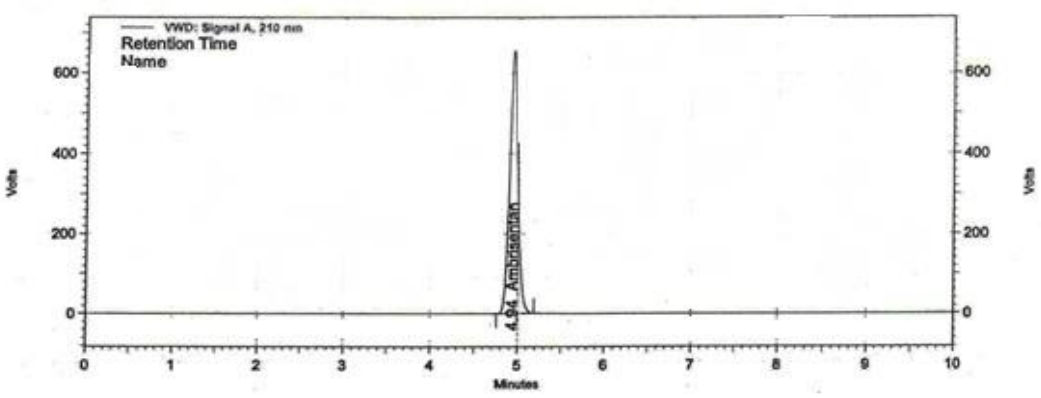


Figure 48: HPLC chromatogram of Ambrisentan for solution stability after 48 hrs

S.No	Name	RT	Area	Plate count	Tailing	Percentage purity
1	Ambrisentan	4.94	75458298	12010	1.22	99.78

Discussion

From the solution stability studies it was found that solution was stable up to 48 hours. The percentage recovery values were found to be 100.34 and 99.78 after 24 and 48 hours respectively.

7.2.9 Assay for Marketed formulation

Preparation of standard solution & sample solution

Sample and standard solution were prepared both as per section 7.1.3 & 7.1.4

Calculation:

The amount of Ambrisentan present in marketed formulation each tablet was calculated by using the following formula:

Amount present

$$= \frac{\text{Sample Area}}{\text{Standard Area}} \text{ X } \frac{\text{Standard weight}}{\text{Standard Dilution}} \text{ X } \frac{\text{Sample Dilution}}{\text{Sample weight}} \text{X} \frac{\text{WS purity}}{100} \text{ X Average weight}$$

$$\textbf{Assay} = \frac{\text{Amount present}}{\text{Label Claim}} \text{ X } 100$$

Average weight of 20 tablets = 91.6 mg.

Weight taken = 461.2 mg.

Label claim: 5 mg of Ambrisentan.

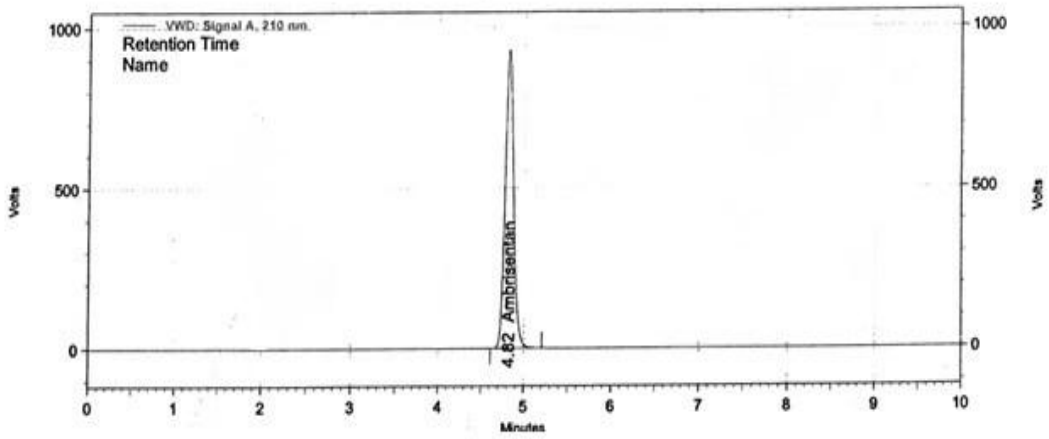


Figure 49: HPLC chromatogram of Ambrisentan for marketed formulation

S. No	Name	RT	Area	Plate count	Tailing
1	Ambrisentan	4.82	74991596	11087	0.91

Table.18 Tabular Column for the Assay of Marketed formulation

S. No	Weight taken	Sample area	Standard area	Content/Tablet in mg	Percentage purity
1	461.2 mg	74991596	75174299	5.01	100.25

Discussion

The percentage purity of Ambrisentan in tablet dosage form was calculated. The percentage purity was found of Ambrisentan was found to be 100.25

8. SUMMARY

On the basis of the experiments, the RP-HPLC method development of Ambrisentan was tried in Kromosil C_{18} column.The various mobile phase compositions were used in different ratios like methanol:water, Acetonitrile:water and Potassium dihydrogenorthophosphatebuffer:Acetonitrile. The various chromatographic conditions were tried to develop a method for analysis. Above all methods were compared and best method was selected for the development and validation of Ambrisentan in tablet dosage form.

Ambrisentan was determined by reverse phase HPLC method using mobile phase A:B (30:70),mobile phase A is phosphate buffer (pH 3.0)and mobile phase B is Acetonitrile:water (90:10) by using a Kromosil ODS C_{18} Column, 5µ (250×4.6mm) as a stationary phase. Detection was carried out using VWD detector at 210 nm. After development of the method, it was validated for system suitability, specificity, linearity, limit of detection and limit of quantification, precision, accuracy, robustness and solution stability studies.

The Specificity of Ambrisentan is shown in Chromatograms. There was no interference in this method and good separation between all peaks. It means no impurity was interfered and also reveals that commonly used excipients and additives present in the tablet dosage form were not interfering in the proposed methods.

From the linearity table.16, it was found that, the drug obeys beer's law. The calibration plot for Ambrisentan was observed as linear in the range of 50-150µg/ml and the correlation coefficient was found 0.999 .From the results shown in the accuracy table17, it was found that recovery values of pure drug from the solution were 98.0 % to 102 %, which indicates that the method is accurate.

The precision was found within the limits. The limits were not more than RSD 2%.Precision RSD was 0.45 for Ambrisentan. This indicates that the method is precise. The data regarding the precision was shown in table18 respectively.

Based on standard deviation response and slope the Limit of detection calculated for Ambrisentan was 0.124 µg/mL and Limit of quantitation calculated for Ambrisentan was 0.370 µg/ml.

In Robustness parameter, the change in flow, temperature, pH and mobile phase composition variation conditions the percentage R.S.D. was found less than 2%. Hence this method was better for method development in small deliberate changes. The data regarding robustness are shown in table 19.

The system suitability was found within the limits. The limit was not more than RSD 2%. The retention time of Ambrisentan was 4.82 min. The data regarding the system suitability are shown in table 20.

During solution stability studies up to 48 hrs the percentage purity of Ambrisentan was within the acceptance criteria of 98-102%

In assay of marketed formulation the percentage purity of Ambrisentan was100.25 which indicates that the method can be used to determine the percentage purity of Ambrisentan in tablet dosage form.

8.2. CONCLUSION

The proposed method was found to be simple, accurate, precise and rapid RP-HPLC method suitable for the estimation of Ambrisentan in tablet dosage form. All the parameters meet the criteria of ICH guidelines for method validation and found to be simple, sensitive, accurate and precise. It can therefore be concluded that the reported method is more economical and can find practical application & may be recommended for routine and QC analysis of the investigated drugs to provide simple, accurate and reproducible quantitative analysis for the determination of Ambrisentan in tablet dosage form.

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